OESTROGEN ACTION IN HUMAN OVARIAN CANCER

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DECLARATION

In accordance with the regulations of the University of Edinburgh, I declare that the work described in this thesis has been composed by myself entirely, except where acknowledgement has been given.

GILLIAN L. HIRST

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ABSTRACT

Ovarian cancer affects over 5000 women each year in the UK and is the most common cause of death from gynaecological malignancy, reflecting the late presentation of the disease. The mechanisms involved in the growth regulation of ovarian cancer are still poorly understood, however epidemiological studies suggest that endocrine factors play an important role in the development and progression of the disease. The majority of ovarian carcinomas possess oestrogen receptors (ER), and several clinical trials suggest that a subset of patients may be responsive to anti-oestrogen therapy. As yet, there have been a limited number of studies investigating the role of oestrogen in the growth regulation of ovarian cancer, partially due to the paucity of defined ER-positive ovarian cancer models.

The aim of this study was to examine the role of oestrogen in a series of ER-positive ovarian cancer cell line models, including the first characterised lines with moderate-high levels of ER. The effects of oestrogen on growth were initially determined in nine ovarian cell lines possessing a range of ER from 0 to 132 fmol/mg protein. Sensitivity to oestrogen correlated with the level of ER expression in that concentrations of 17 β-oestradiol (E_2) between 10^{-12} and 10^{-6}M stimulated the growth of the PEO1, PEO4 and PEO6 cell lines which possess moderate-high levels of receptors between 96 and 132 fmol/mg protein, whereas there was no change in the growth of the PEA1 and PEA2 cell lines which have an ER content of less than 30 fmol/mg protein. The growth of the ER-negative lines PEO14, PEO16 and PEO23 was also unchanged by E_2 treatment. Concentrations of E_2 which were stimulatory to

the PEO1 cell line were inhibitory to a cisplatin-resistant derivative, $PEO1^{CDDP}$ despite this also possessing moderate-high levels of ER. Effects of E_2 on growth were also examined in two ovarian xenograft models grown in nude mice; PEO4 and HOX60 which are ER-positive and ER-negative respectively. Exposure of the xenografts to a subcutaneously planted oestrogen pellet produced a significant inhibition in PEO4 growth but no difference in HOX60 growth as compared to controls.

ER levels in the ER-positive PEO1, PEO4 and $PEO1^{CDDP}$ cell lines were down-regulated by treatment with 10^{-10}M E_2, whereas there was no change in the receptor status of the PEA1, PEA2 or ER-negative lines. Levels of progesterone receptors (PR), shown to be a marker of oestrogen sensitivity in breast cancer, were up-regulated by the same concentration of E_2 in the ER-positive PEO4 and PEO6 cell lines but not in $PEO1^{CDDP}$, PEA1, PEA2 or the ER-negative ovarian lines. In the PEO4 xenograft, ER levels were also reduced and PR levels up-regulated by E_2, but no differences in receptor levels were observed in the ER-negative HOX60. There was only a low level of expression of the oestrogen-inducible protein pS2, in both the ER-positive and ER-negative ovarian cell lines and this was not altered following E_2 treatment. Expression of HSP27, another oestrogen-regulated protein, appeared to correlate with ER status and oestrogen sensitivity, being highest in the PEO1, PEO4 and PEO6 cell lines, and lower in the PEA1, PEA2 and ER-negative lines. This was also observed *in vivo*; HSP27 expression in the PEO4 xenograft was significantly higher than that seen in HOX60. After exposure to 10^{-10}M E_2 HSP27 expression was significantly down-regulated in the ER-positive PEO4 and $PEO1^{CDDP}$ lines, but

unaltered in ER-negative PEO14 cells. No change in expression was observed following E_2 treatment of the PEO4 xenograft.

To determine whether oestrogen exerted its growth effects on the ER-positive cell lines through the modulation of growth factors, the production of TGF-α and EGF was measured in conditioned medium from cells with or without exposure to 10^{-10}M E_2. Levels of TGF-α secreted by PEO4 and $PEO1^{CDDP}$ cells were significantly higher following E_2 treatment as compared to controls, whilst levels were unchanged in PEO14 cells. No detectable levels of EGF were found in any of the conditioned media. E_2 treatment also up-regulated the expression of IGF-I receptors in the ER-positive PEO4 cell line.

If these results reflect the clinical situation, then there is a valid case for the use of anti-oestrogen therapy in a subset of patients with ovarian tumours expressing moderate-high levels of ER. PR and HSP27 expression levels may help indicate which tumours are hormonally sensitive, and thus more likely to respond to treatment. Agents which interfere with the TGF-α and IGF-mediated growth pathways may also have therapeutic benefit.

ABBREVIATIONS

BRCA1	Breast-ovarian cancer susceptibility gene 1
BSA	Bovine serum albumin
CARL	Composition adjusted receptor level
CDDP, cisplatin	Cis-diamminedichloroplatinum (II)
° C	Degrees Celsius
cpm	Counts per minute
dH_2O	Distilled water
dcsFCS	Double charcoal stripped foetal calf serum
DES	Diethylstilbestrol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
ER	Oestrogen receptor
ERE	Oestrogen response element
E_2	17 β-oestradiol
FACS	Fluorescence-activated cell sorter
FIGO	International Federation of Gynaecology and Obstetrics
FITC	Fluorescein isothiocyanate
fmol	Femtomole, 10^{-15}M
FSH	Follicle-stimulating hormone
g	Gram (s) / centrifugal field
GPUT	Galactose-1-phosphate uridyl transferase
HNPCC	Hereditary non-polyposis colorectal cancer
HSE	Heat shock element
HSF	Heat shock factor
HSP27	Heat shock protein 27
IGF-I	Insulin-like growth factor I
IGF-I R	Insulin-like growth factor I receptor
IGFBP	Insulin-like growth factor binding protein
IU	International units
kb	Kilobase
kDa	Kilodaltons
LDL	Low density lipoprotein
LHRH	Luteinizing hormone releasing hormone
MAP kinase	Mitogen-activated protein kinase
MDR	Multi-drug resistance
μM	Micromole
μg	Microgram(s)
ml	Millilitre
MPA	Medroxyprogesterone acetate
mRNA	Messenger ribonucleic acid
mw	Molecular weight
OD	Optical density
OHT	4-hydroxytamoxifen
OPD	*O*-phenylene diamine hydrochloride
PBS	Phosphate-buffered saline

pg	Picogram(s)
PMSF	Phenyl methylsulphonyl fluoride
PSP	Porcine spasmolytic polypeptide
PR	Progesterone receptor
RIA	Radioimmunoassay
rpm	Revolutions per minute
RPMI 1640	Roswell Park Memorial Institute 1640 (Media)
SEM	Standard error of the mean
TGF-α/β	Transforming growth factor alpha/beta
TMB	Tetramethylbenzidine
TPA	12-O-tetradecanoyl-phorbol 13-acetate
w/v	Weight for volume
v/v	Volume for volume
WHO	World Health Organisation

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1. INTRODUCTION

1.1 Introduction

It has long been known that hormones, and in particular oestrogens may play a part in the progression of certain cancers. A relationship between ovarian function and malignant breast disease was noted almost one hundred years ago by Sir George Beatson who successfully healed a locally recurrent cancer of the breast following ovariectomy (Beatson, 1896). There is also evidence to implicate hormones in the development of ovarian cancer, and the aims of this thesis are to investigate the role of oestrogen in the biology of this carcinoma.

Evidence for endocrine involvement in ovarian cancer is discussed in the following introduction which is divided into two main parts. The first section provides a general background, and the second is concerned with growth regulation.

1.2 Ovarian Cancer

1.2.1 Incidence

Ovarian cancer is one of the most common gynaecological malignancies, with approximately 5000 women being diagnosed per year in the UK (MRC Gynaecological Working Party, 1990). It has the highest mortality rate of all gynaecological cancers (85% will die from their disease) and is the fifth most common cause of cancer related death in women. Part of the reason for this poor prognosis is the rapid and asymptomatic progression of the tumour such that it usually presents as late stage disease. Ovarian cancer can occur at any age but

incidence of the disease appears to increase around the age of 45, peaking at 70-80 years so that the majority of cases are detected in post-menopausal women (Brinton and Hoover, 1992, Yancik, 1993). Less than 15 cases per 100, 000 per year occur in those under 45.

1.2.2 Presentation and staging of disease

Abdominal pain and swelling are the most obvious signs of disease at initial presentation. This occurs due to expansion of the tumour, adherence to surrounding tissue and formation of ascites. A diagnosis may be assisted by ultrasonography which has been developed as a pre-operative guide (Andolf *et al.*, 1986). Ascites as well as tumours can be detected by this method. Pleural effusions can be demonstrated by chest radiography, and lymphatic spread by lymphography. Tumour markers such as CA-125, a high molecular weight mucin glycoprotein, may also aid diagnosis. This antigen may be present on the surface of both benign and malignant ovarian tumours (Niloff *et al.*, 1984) but is often absent or only weakly expressed by normal ovarian surface epithelia (reviewed in Auersperg *et al.*, 1996). Levels of circulating CA-125 are elevated in 80% of ovarian cancers (Bast *et al.*, 1983). Currently, it is used as a marker for monitoring response to therapy and relapsed disease. In this context it has been shown to have prognostic use, predicting macroscopic disease recurrence by three months on average (Niloff *et al.*, 1986).

Work by Zurawski *et al.*, (1988) and others have indicated that CA-125 may have utility in screening for ovarian cancer, showing that 50% of the population had elevated CA-125 levels eighteen months before clinical appearance of the disease.

However, two recent interventional studies suggest that elevated CA-125 levels alone are not sensitive enough as a predictive marker for ovarian cancer. Einhorn *et al.*, (1992) showed that in a population screen of 5,550 women, 2% over 50 years had elevated levels. These women and a similar number of controls were followed up for further study incorporating 3-monthly CA-125 assessment and 6-monthly examination and ultrasonography. Six cases of ovarian cancer were detected in this study but three cases were missed by screening. In another large study, 11 ovarian cancers were detected by a CA-125 screen but a further 18 were not identified (Jacobs *et al.*, 1993).

Staging of disease as determined at surgery (laparotomy) is of prognostic value in determining treatment strategy (Carey *et al.*, 1993). Definitions of the different stages are given in Table 1.1. The inability to detect cancers early due to lack of adequate screening procedures and absence of clinical symptoms means that advanced stage disease has a poor prognosis. Patients with stage I disease have the best outcome with a 5 year survival rate of 80-90%. Stage II disease has a poorer outcome with a 5 year survival rate of 40-60%. The poorest prognoses are observed with stage III (10-15%) and stage IV disease, this having a 5 year survival rate of less than 5% (Friedlander and Dembo, 1991).

Table 1.1

FIGO (International Federation of Gynaecology and Obstetrics) staging for carcinoma of the ovary, 1985

Stage I		Growth limited to the ovaries
Stage Ia		Growth limited to one ovary
	(i)	No tumour on the external surface; capsule intact
	(ii)	Tumour present on the external surface; and/or capsule ruptured.
Stage Ib		Growth limited to both ovaries; no ascites.
	(i)	No tumour on the external surface; capsule intact
	(ii)	Tumour present on the external surface; and/or capsule ruptured.
Stage Ic		Tumour at either stage Ia or Ib, but with tumour on surface of one or both ovaries; or with capsule ruptured; or with ascites present containing malignant cells or with positive peritoneal washings.
Stage II		Growth involving one or both ovaries with pelvic extension.
Stage IIa		Extension and/or metastases to the uterus and/or fallopian tubes.
Stage IIb		Extension to other pelvic tissues.
Stage IIc		Tumour at either stage IIa or IIb, but with tumour on surface of one or both ovaries; or with capsules ruptured; or with ascites present or peritoneal washings.
Stage III		Growth involving one or both ovaries with intraperitoneal metastases outside the pelvis and/or positive retroperitoneal nodes or inguinal nodes. Superficial liver metastases equals stage III. Tumour limited to the true pelvis, with histologically confirmed extension to the small bowel or omentum.
Stage IIIa		Tumour grossly limited to the true pelvis with negative nodes but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces.
Stage IIIb		Tumour involving one or both ovaries with histologically confirmed implants of abdominal peritoneal surface, none exceeding 2cm in diameter. Nodes are negative.
Stage IIIc		Abdominal implants greater than 2cm in diameter and/or positive retroperitoneal or inguinal nodes.
Stage IV		Growth involving one or both ovaries, with distant metastases or pleural effusion is present, with positive cytology or metastasis to the liver parenchyma.

1.2.3 Pathology

Ovarian neoplasms have a varied histology but can be classified into several groups depending upon their origin (Figure 1.2), and may be either benign, borderline (those with low malignant potential) or malignant. The most frequently observed ovarian carcinomas originate from the surface epithelium of the ovary and account for over 90% of all malignant ovarian neoplasms. These are subcategorised into serous, mucinous, endometrioid, clear cell, undifferentiated adenocarcinomas and the rare Brenner tumour.

The different epithelial types portray similar histological characteristics to the tubal, endometrial and endocervical derivatives of the Mullerian ducts which develop from the primative mesothelium in the embryo. The ovarian epithelial surface is the adult equivalent of this mesothelium and neoplasms arising from this retain their embryonic potential to differentiate. Thus, serous tumours resemble the epithelium of the fallopian tube, endometrioid and clear cell tumours the endometrium, and mucinous tumours the endocervical epithelium. The origin of Brenner tumours is different in that they shows histology similar to that of uro-epithelium and are thought to exhibit Wolffian (mesonephric), rather than Mullerian, differentiation (Roth, 1971, Cummins *et al.*, 1973).

Table 1.2

Summary of Malignant Ovarian Tumour Classification according to WHO (Serov and Scully, 1973)

I	Common 'epithelial' tumours
	A. Serous tumours
	B. Mucinous tumours
	C. Endometrioid tumours
	D. Clear cell (mesonephroid) tumours
	E. Brenner tumours
	F. Mixed epithelial tumours
	G. Undifferentiated carcinoma
	H. Unclassified tumours
II	Sex cord stromal tumours
III	Lipoid cell tumours
IV	Germ cell tumours
V	Gonadoblastoma
VI	Soft tissue tumours not specific to the ovary
VII	Unclassified tumours
VIII	Secondary (metastatic) tumours
IX	Tumour-like conditions

The most common type of malignant epithelial ovarian tumours are serous cystadenocarcinomas, representing about 40-60% of cases. Tumours of the mucinous subtype constitute about 3 to 21%, whilst endometrioid and clear cell subtypes account for 5 to 20% and 5 to 10% respectively (reviewed in Slotman and Rao, 1988). Presentation of other malignant epithelial tumour types is rare. The degree of tumour differentiation has also been categorised and should be included in a full

histopathological diagnosis (Baak *et al.*, 1987). Grade 1 classifies a well-differentiated tumour exhibiting little cellular atypia and few mitoses. Moderately-differentiated tumours demonstrating increasing cellular atypia and more frequent mitoses but retention of histological differentiation are classed as Grade 2, and Grade 3 tumours are the most aggressive, being poorly-differentiated with frequent mitoses, and containing layers of poorly or undifferentiated cells with few distinguishing features making histological classification difficult.

1.2.4 Epidemiology

The cause of ovarian cancer is poorly understood despite extensive research into environmental, endocrinological and genetic factors. Further insight into the epidemiology and etiology of this carcinoma may aid prevention and earlier diagnosis.

(i) Endocrine factors

There is considerable evidence to suggest that hormones may be implicated in the progression of this disease. Ovarian cancer incidence has been linked with breast cancer which is generally thought to be hormonally influenced. Thus, a woman with a primary breast carcinoma is twice as likely to develop ovarian cancer, and a patient with ovarian cancer is four times as likely to develop breast cancer (reviewed in Hamilton, 1992). Further, the two cancers have etiological factors in common. For example, early menarche, late menopause, and nulliparity appear to be associated with an increased risk of ovarian cancer, whereas a decreased risk is linked with pregnancy (Cramer *et al.*, 1983a, Franceschi *et al.*, 1991, Polychronopoulou *et al.*, 1993). The increase in the incidence of ovarian cancer above the age of 45 is thought to be

associated with higher levels of gonadotropins which may contribute to carcinogenesis (Cramer and Welch, 1983b). Numerous studies have also indicated a reduced risk with the use of oral contraceptives which suppress ovulation (Stanford, 1991, Rosenblatt *et al.*, 1992). Long term use (five years or more) is associated with a 50% reduction in ovarian cancer risk, and this protective effect appears to persist ten or more years after use is discontinued (Vessey and Painter, 1995).

Conversely, an increased risk is associated with women taking oestrogens for non-contraceptive use. Rodrigez *et al.*, (1995) have recently published the results of a large study indicating increased ovarian cancer risk of 40% with 6-10 years of oestrogen replacement therapy, and 70% with greater than 10 years of treatment. There is also evidence to implicate the use of fertility drugs with a higher incidence of ovarian cancer. A small study by Ron *et al.*, (1987) showed no increased risk of ovarian cancer in women who had used fertility drugs, although an increased risk of endometrial carcinoma was noted. However, a more recent report has demonstrated a 2.5 fold increase in ovarian cancer risk associated with the use of fertility drugs such as exogenous gonadotropins and pituitary gonadotropin stimulants (Rossing *et al.*, 1994).

Evidence for the involvement of hormones in the progression of established ovarian cancer will be covered in section 1.3 under growth regulation.

(ii) Dietary and environmental factors

Ovarian cancer incidence is higher in industrialised countries with the exception of Japan. However in a pattern that is seen with many cancers, migrant Japanese women

and their offspring in the United States exhibit an increased frequency of ovarian neoplasms compared to Japanese women living in Japan (Herrinton *et al.*, 1994). Dietary factors and environmental carcinogens are thought to be contributory elements. A report by Cramer *et al.*, (1984) proposed an associated risk with animal fat consumption. Subsequently, a large case-control study into ovarian cancer risk and diet has suggested that for every 10g of saturated fat ingested per day, the risk of ovarian cancer increases by 20%, whereas the same weight of vegetable fibre confers a 37% reduced risk (Risch *et al.*, 1994). Oestrogens and other steroid hormones are often present in saturated fat (Risch *et al.*, 1994) and high fibre diets have been shown to reduce serum oestrogen concentrations in premenopausal women (Rose *et al.*, 1991, Goldin *et al.*, 1994). However, more studies are needed to confirm these reports.

There is no evidence to suggest that smoking may be a contributory factor in increasing ovarian cancer risk (Whittemore *et al.*, 1988). Likewise, several studies have reported no increased ovarian cancer incidence in alcohol drinkers, with two studies suggesting a protective effect of alcohol in young women (Gwinn *et al.*, 1986, reviewed in Boyle and Leake, 1996). Coffee drinking has been ascribed with a slight increase in risk (Byres *et al.*, 1983, Cramer *et al.*, 1984, Whittemore *et al.*, 1988).

Two possible chemical carcinogens suggested to have some association with ovarian cancer incidence are asbestos and talc (Henderson *et al.*, 1979, Longo and Young, 1979). Talc until recently contained asbestos which is known to be involved in the development of malignant mesothelioma and lung cancer. Evidence by Cramer *et al.*,

(1982) supports a link between talc use and ovarian cancer, however other workers do not confirm this observation (Whittemore *et al.*, 1988, Harlow *et al.*, 1992, Wehner, 1994).

A case/control study by Cramer *et al.*, (1989) showed that higher consumption of lactose/galactose in yoghurt and cottage cheese was associated with lower levels of galactose-1-phosphate uridyl transferase (GPUT) in ovarian cancer cases as compared with controls, and there is some evidence to suggest that GPUT may be a genetic risk factor for early menopause (Cramer, 1990). However, a recent large Canadian case study has shown that average daily intake of lactose or galactose, or lactose intolerance is not associated with risk of ovarian cancer (Risch *et al.*, 1994).

(iii) Genetic factors

For the majority of ovarian cancer patients there is no family history of the disease. However it is estimated that 5% of cases are hereditary, the majority of these being of the papillary serous subtype (Hamilton, 1992). Three categories of familial ovarian cancer have been identified (Lynch *et al.*, 1978, Piver *et al.*, 1984). The first is site-specific disease which is the most common of the three and involves an increased risk of only ovarian cancer. The second instance is where there is increased risk of associative ovarian cancer in familial breast cancer cases, the breast-ovarian cancer syndrome. The final circumstance is the cancer family syndrome (Lynch Syndrome II) which integrates hereditary non-polyposis colorectal cancer (HNPCC) (Lynch Syndrome I) with risk of gynaecological (ovary, endometrium), gastrointestinal, urological and breast cancers (Lynch *et al.*, 1985).

Familial cancer tends to arise at a relatively early age, around 35 to 40 years, compared with sporadic cases occuring at a median age of 61 years (Ozols *et al.*, 1992). It is inherited in an autosomal dominant pattern with high penetrance such that up to 50% of first degree relatives may inherit the gene (Piver *et al.*, 1991). Interestingly, in site-specific disease, males may convey predisposition of the gene to their female offspring, but they and any male progeny do not appear to have any increased risk of any form of cancer (Piver *et al.*, 1991, Lynch *et al.*, 1990). It was suggested that the gene involved may be associated with the growth regulation of only the ovarian epithelial cell type (Hamilton, 1992).

The majority of patients with breast/ovarian cancer syndrome show linkage to a predisposing gene (BRCA1) (Easton *et al.*, 1993) which was mapped to the long arm of chromosome 17, in the region 17q12-21 (Hall *et al.*, 1990, Narod *et al.*, 1991). The gene was cloned and characterised by Miki and coworkers, (1994), and shown to encode a zinc finger protein of unknown function. Characterisation of mutations suggest that BRCA1 may be a tumour suppressor gene (Futreal *et al.*, 1994, Castilla *et al.*, 1994, Takahashi *et al.*, 1995). The majority of hereditary site-specific ovarian cancers have also been linked to BRCA1 (Steichen-Gersdorf *et al.*, 1994).

Recent observations imply that BRCA1 is involved in the proliferation and differentiation of mammary tissue under the influence of ovarian hormones. Marquis *et al.*, (1995), and Gudas *et al.*, (1995) have shown an increase in BRCA1 mRNA expression in breast cancer cells following treatment with oestrogen.

Mutations in a second gene BRCA2, which has been mapped to chromosome 13q12 proximal to the retinoblastoma gene (Wooster *et al.*, 1994), are also associated with an excess of ovarian cancers in members of affected families (Narod *et al.*, 1995) and have been reported to confer a higher risk of male breast cancer. Current observations suggest that other genes are likely to be involved in the remaining cases of inherited ovarian cancer.

Several predisposing genes for Lynch Syndrome I and II have been cloned recently (hMSH2, hMLH1, hPMS2 and hPMS1), and shown to encode a family of proteins whose normal function is involved in post-replication mismatch repair (Fishel *et al.*, 1993, Kolodner *et al.*, 1995, Nicolaides *et al.*, 1994). Cells with defects in these genes have been shown to possess a 'mutator phenotype', which is often seen as mutations in repetitive sequences (microsatellite instability). This has been observed in heritable forms of ovarian carcinomas, but is not typically found in sporadic cases (Wooster *et al.*, 1994).

Mutations of the p53 tumour suppressor gene, allele loss and aberrent expression of the p53 protein have been reported in about 30-50% of ovarian cancer cases and appear to be associated with serous histology and advanced stage (Marks *et al.* 1991, Eccles *et al.*, 1992, Milner *et al.*, 1993, Runnebaum *et al.*, 1994). Mutations are well dispersed throughout the p53 gene and seem to be mainly transitions (Kohler *et al.*, 1993).

Aberrant expression of several oncogenes including c-*erb*B-2 (*HER-2/neu*) which codes for an epidermal growth factor receptor-like protein, *ras*, *myc*, *fms*, *jun* and

myb has been described in ovarian cancer (reviewed in Berek and Martinez-Maza, 1995). Several studies have shown c-*erb*B-2 to be overexpressed in about 20-30% of ovarian cancers investigated and associated with poor survival (Slamon *et al.*, 1989, Berchuck *et al.*, 1990a, Rubin *et al.*, 1994). Amplification/activation of K-*ras*, and occasionally H-*ras* and N-*ras*, has been seen in approximately 15% of mainly advanced ovarian carcinomas (Bolz *et al.*, 1989, Liehr *et al.*, 1993, Yokota *et al.*, 1986), and a higher proportion of tumours demonstrate overexpression of c-*myc*, usually accompanied with amplification of the gene (Kohler *et al.*, 1989, Tashiro *et al.*, 1992, Liehr *et al.*, 1993).

1.2.5 Treatment

(i) Surgery

The main aims of surgery for primary ovarian cancer are diagnosis, staging and reduction of tumour burden. With early stage disease (ie. stage Ia/b), surgery can remove the whole tumour and represent adequate treatment (Young *et al.*, 1990). Any subsequent recurrence probably develops from occult micrometastases or implantation of free tumour cells from the peritoneal fluid (Piver *et al.*, 1978). The surgical procedure in early disease usually consists of tumour removal followed by bilateral salpingo-oophorectomy and total abdominal hysterectomy (Hudson, 1973).

In the treatment of advanced disease, incomplete erradication of tumour, termed cytoreductive surgery, is performed in 30-50% of cases. This helps to ease discomfort and reduce tumour effects on metabolism. It also improves the chances of response to adjuvant chemotherapy. Size of remaining tumour is the most important prognostic

factor in advanced disease (Griffiths and Fuller, 1978, Wharton and Herson, 1981, Cannistra, 1993).

A second-look laparotomy may be performed to assess patients who are clinically disease free after a course of chemotherapy (reviewed in Podratz and Kinney, 1993). It may also aid further cytoreductive surgery and restaging of disease. A recent study by Van der Burg *et al.*, (1995) showed that a second debulking procedure in patients with advanced stage III and IV (who after initial debulking had residual disease $\geq$ 1cm followed by 3 courses of chemotherapy), significantly improved the survival of patients.

(ii) Chemotherapy

Whilst surgery may be adequate for early stage Ia disease, consideration has to be given to additional systemic treatment in more advanced tumours. Single alkylating drugs such as melphalan, chlorambucil and cyclophosphamide have been widely used for patients with advanced disease since the 1960's and represented the first successful approach to the treatment of this carcinoma with response rates of 40-60% (Tobias and Griffiths, 1975, reviewed in Sutton, 1994). They act by generating carbonium ions which bind to DNA causing crosslinking and single strand breaks. Non-alkylating drugs such as doxorubicin and hexamethylmelamine have also been implemented as single agents with similar response rates (De Palo *et al.*, 1977, Weiss, 1981).

Current standard chemotherapy usually consists of cisplatin-containing regimens, most usually with an alkylating agent such as doxorubicin, which have been shown to

produce greater response rates than cisplatin alone (Levin *et al.*, 1993). Cisplatin analogues elicit cell death by forming interstrand and intrastrand crosslinks within DNA at amino or hydroxyl groups, the major lesions being intrastrand crosslinks between N-7 atoms of adjacent purines (Sherman and Lippard, 1987). The presence of these adducts leads to a change in DNA conformation (Anin and Leng, 1990), which may cause a block in DNA replication, possibly generating DNA double strand breaks which are a signal for apoptosis (Nelson and Kastan, 1994). Carboplatin, an analogue of cisplatin, has been shown to be an effective and less toxic substitute in drug treatment regimens in advanced ovarian cancer. It is less nephrotoxic, neurotoxic and emetogenic than cisplatin (Wiltshaw *et al.*, 1984, Mangioni *et al.*, 1989), and in combination chemotherapy demonstrates similar response rates to cisplatin-containing regimens (Alberts *et al.*, 1992, Swenerton *et al.*, 1992) although some evidence points to it being less effective in terms of survival (Vermorken *et al.*, 1993).

In the mid 1980's, clinical trials began with a new series of compounds, the taxanes. The drug taxol (Paclitaxel), was first isolated in the 1960's from a crude extract of the bark of the Western Pacific yew tree, *Taxus brevifolia*, as part of the US National Cancer Institute's screening programme, and was found to have activity against several murine tumours (Wani *et al.*, 1971). Taxol and docetaxel (Taxotere), a semisynthetic analogue obtained from the European yew, are currently the two compounds in clinical use. Taxanes act as mitotic inhibitors by promoting the polymerisation and inhibiting the depolymerisation of tubulin which is a component of the microtubules responsible for spindle formation in mitosis (reviewed in Gore,

1996). The microtubules become stable thus blocking cells in late G_2 and/or M phases of the cell cycle (Schiff *et al.*, 1979). Taxol and docetaxol appear to be promising second line treatments for ovarian cancer refractory to other drugs with response rates of around 30% in patients who have failed to respond to prior cisplatin therapy (Einzig *et al.*, 1992, Kavanagh *et al.*, 1993, Thigpen *et al.*, 1994). There is little data on the action of taxol as a single agent in previously untreated patients with ovarian cancer. A report from an ongoing study suggests that the response rate in 28 patients (32%) is slightly lower than expected (Gore *et al.*, 1995). However, as part of combination chemotherapy with cisplatin in first line treatment, taxol is reported to increase survival to 37.5 months versus 24.4 months in patients receiving cyclophosphamide plus cisplatin (McGuire *et al.*, 1995).

(iii) Drug resistance

Since the introduction of cisplatin in the 1970's, and carboplatin in the 1980's the survival of patients with advanced ovarian cancer has increased significantly (Advanced Ovarian Trialists Group, 1991). Comparisons in mortality data from 1975 and 1985 in the West of Scotland show an increase in 3-year survival from 36% to 50% for women under 55 (Gillis *et al.*, 1991). However, in the majority of studies the median survival of patients treated with cisplatin-based regimens is approximately 21 to 30 months. Chemotherapy frequently produces complete clinical remissions but relapse is a common event and when clinical drug resistance occurs it is usually fatal (reviewed in Hamilton *et al.*, 1995, Kaye, 1996). One possibility is altered host pharmacology, but it is more likely to be due to a combination of both intrinsic and acquired mechanisms; an outgrowth of intrinsically resistant cells or repeated

exposure to drugs altering the target cells and rendering them refractory to further administrations of drug. In the study of cisplatin resistance, investigations using *in vitro* cell lines have identified several mechanisms which may be involved: defective drug transport, increased levels of intracellular metallothioneins, glutathione or glutathione-S-transferase activity which may inactivate drugs, and enhanced repair of DNA damage (Andrews and Howell, 1990, Gately and Howell, 1993). Mutations in the p53 gene may be associated with drug resistance. A recent report suggests that defects in p53 are associated with resistance to carboplatin (Al-Azraqi *et al.*, 1994) and several studies suggest that p53 is necessary for certain drugs, including cisplatin to induce an apoptotic response (Lowe *et al.*, 1993, Clarke *et al.*, 1993, reviewed in Brown, 1996). Furthermore, changes in oncogene expression may be implicated in drug resistance. Blocking of c-*erb*B-2 overexpression with monoclonal antibodies results in enhanced cytotoxicity of cisplatin in ovarian cancer cells (Hancock *et al.*, 1991) and a similar effect is noted with antibodies directed against the epidermal growth factor (EGF) receptor (Christen *et al.*, 1990). These may work by blocking DNA repair (Pietras *et al.*, 1994). Other studies implicate the overexpression of the efflux pump P-glycoprotein, which is involved in the classic multidrug resistant phenotype (MDR), in being partly responsible for the reduced cytotoxicity of drugs such as doxorubicin in human ovarian cancer cell lines (Bradley *et al.*, 1989, Maeda *et al.*, 1993). Use of MDR modulators has been shown to restore the taxol sensitivity of multidrug resistant cells (Jachez *et al.*, 1993).

These studies *in vitro* have now led to the use of some potential resistant modulators for ovarian cancer in clinical trials, which include buthionine sulfoximine (BSO) which

causes depletion of glutathione (O'Dwyer *et al.*, 1992), aphidicolin, a repair inhibitor (Sessa *et al.*, 1991) and PSC 833, an MDR modulator in conjunction with taxol (Fisher *et al.*, 1994).

(iv) Radiotherapy

Recently, interest has been renewed in the use of radiotherapy for the treatment of ovarian cancer due to the poor survival rate of patients even after a negative second look laparotomy. However, several studies have shown that the only real benefit from whole abdominal irradiation appears to be in patients with microscopic disease (Bruzzone *et al.*, 1990, Lederman *et al.*, 1991).

(v) Hormonal therapy

Tamoxifen, the synthetic anti-oestrogen employed widely in the treatment of breast cancer has also been used in the treatment of ovarian cancer. The first report of its use was in 1981 where responses were noted in two patients (Myers *et al.*, 1981). Following this, a number of small studies reported response rates of around 10%. However, stabilization of disease was noted in several studies (Landoni *et al.*, 1983, Shirey *et al.*, 1985, Weiner *et al.*, 1987). More recently, studies have shown a 10% complete response rate, and a 7% partial response rate in 105 patients with stage III/IV disease which was considered chemoresistant (Hatch *et al.*, 1991) and an 18% response rate to tamoxifen in 29 patients with refractory stage III/IV disease which included two complete responses (Ahlgren *et al.*, 1993). Abrogation of platinum resistance has been observed *in vitro* when tamoxifen is added concomitantly

however the underlying mechanism is still unresolved (McClay *et al.*, 1992, Nakata *et al.*, 1995).

Progestins have been used in a variety of studies for the treatment of ovarian cancer with the rationale that they may counteract the growth promoting effects of oestrogens. Objective response rates of only about 10-15% have been observed in most studies (reviewed in Slotman and Rao, 1988) but using medroxyprogesterone acetate (MPA) Rendina *et al.*, (1982) reported a response rate of 55%, although the majority of tumours treated had a favourable prognosis being of the endometrioid subtype and well-differentiated. Additionally, progestins have been used in combination with other agents such as alkylating drugs. Postoperative treatment with MPA and melphalan yielded response rates of 85% (Bergqvist *et al.*, 1981) and Guthrie, (1979) recorded complete responses in 46% of patients. These response rates could not be repeated in three prospective controlled studies where chemotherapy plus MPA was compared with chemotherapy alone (reviewed in Slotman and Rao, 1988). In two trials with sequentially administered hormonal therapy consisting of oestrogen treatment (to induce progesterone receptors, PR) followed by a progestin, 14-17% response rates were produced (Freedman *et al.*, 1986, Fromm *et al.*, 1991).

Luteinizing hormone releasing hormone (LHRH) receptors are reported to be present in about 80% of ovarian cancers (Emons and Schally, 1994) and another therapeutic approach has employed gonadotropin-releasing hormone analogues to treat patients with refractory ovarian cancer. Response rates and disease stabilization in about

10-50% of patients treated with LHRH agonists have been reported (Emons *et al.*, 1992, Ron *et al.*, 1995).

An important consideration that should be borne in mind is that whilst low response rates have been observed in these studies, (about one in six patients respond), these trials were performed after failure of other treatment strategies, thereby selecting a subgroup of patients with poor prognosis. In addition, most of the studies did not make use of tumour estrogen or progesterone receptor status. When selecting endocrine therapy in breast cancer, the predisposition of tumours to hormonal control is a most important parameter and receptor measurements are routinely performed in the management of this disease. If markers of hormonal response are assessed in ovarian cancer patients, it may help define a set of oestrogen-sensitive tumours.

1.2.6 Prognostic factors in ovarian cancer

Defining prognostic markers in tumours helps characterize certain subgroups of patients who then can be optimally treated according to this assessment. In breast cancer the identification of several predictive markers has helped to define a subset of hormonally responsive tumours which will respond to endocrine therapy. Further markers are being characterised.

One of the most useful prognostic indicators in ovarian cancer is tumour stage, the more advanced the disease the worse the outcome. Several other factors which have utility include tumour histology, patient age and residual tumour size after surgery (de-Souza and Friedlander, 1992). In general mucinous, endometrioid and clear cell tumours appear to have the best outcome (reviewed by Slotman and Rao, 1988). In

addition, well-differentiated tumours tend to be associated with lower stage disease. As mentioned previously, a better response to chemotherapy is observed in patients with minimal residual disease after cytoreductive surgery. This is the most important prognostic factor for patients with advanced disease (Wharton *et al.*, 1981).

Analysis of tumour DNA content has also been shown to be a strong independent prognostic factor. Rates of aneuploidy are high in ovarian cancer and generally are associated with poor prognosis (Brescia *et al.*, 1990, Friedlander *et al.*, 1984) although other studies do not agree (Sahni *et al.*, 1989, Schneider *et al.*, 1990).

C-*erb*B-2 is overexpressed in about a third of ovarian cancers but some doubt remains as to its prognostic significance (Haldane *et al.*, 1990, Rubin *et al.*, 1993). As mentioned previously, p53 mutations are associated with serous histology and tumours of advanced stage which have a poor prognosis (Niwa *et al.*, 1994, Eccles *et al.*, 1992), however there is some debate as to p53 status being an independent marker (Sheridan *et al.*, 1994, Frank *et al.*, 1994). A recent study suggests that patients with normal sequence p53 are more likely to respond to chemotherapy than those with mutations in the gene (Al-Azraqi *et al.*, 1995).

Investigation of markers of hormonal sensitivity in ovarian cancer may not only help determine the role of oestrogen in this disease but identify tumours which may respond to endocrine therapy, as in the case for breast cancer where the prognostic significance of oestrogen responsive proteins such as oestrogen and progesterone receptor, pS2 and Cathepsin D has been widely reported. These and other markers of

hormonal sensitivity are discussed in further detail in the next section 1.3.1, under Endocrine Regulation.

1.3 GROWTH REGULATION

The presence of specific receptors for hormones and growth factors suggests that the growth of ovarian cancer cells may depend upon these agents. These factors have been shown to be involved in the growth of breast cancer cells and the evidence that ovarian cancer may be similarly regulated is discussed in detail below.

1.3.1 Endocrine regulation

(i) Introduction

As mentioned previously, epidemiological data suggests that hormones are involved in the development of ovarian cancer. This evidence lends support to two hypotheses for the possible development of this carcinoma: the 'gonadotropin hypothesis' and the 'incessant ovulation hypothesis' (reviewed in Piver *et al.*, 1991). The first theory speculates that a continuously high level of gonadotropins resulting from either ovarian failure or a block in the regulatory ovarian/pituitary feedback mechanism, acts on the ovary and leads to tumourigenesis (Gardner, 1948). At menopause the levels of gonadotropins are high but then decline and the age-incidence of ovarian cancer may reflect this pattern. Both pregnancy and the use of oral contraceptives lower gonadotropin levels and suppress ovulation, and have been shown to reduce the risk of ovarian cancer (Cramer *et al.*, 1983a). The 'incessant ovulation theory' proposed by Fathalla in 1971 postulates that continuous trauma to the ovaries caused by

ovulation leads to the development of cancer. The disruption of the surface epithelium after ovulation is repaired by rounds of cell division. It is thought that this continual requirement for growth activity markedly increases the probability of malignant transformation. Mutations are much more likely to occur in dividing as opposed to quiescent cells. Most other mammals exercise their reproductive potential to the full and experience few ovulatory cycles, but in humans ovulatory cycles are experienced almost continuously from puberty to menopause (Fathalla, 1971). Experimental support for this hypothesis comes from studies in hens in which frequent ovulatory activity (induced by artificial lighting) was associated with almost a 100% incidence of ovarian cancer as compared to no incidence in hens kept in normal lighting which ovulate seasonally (Wilson *et al.*, 1958). Ovarian cancer is thought to arise most frequently in surface epithelial inclusion cysts resulting from the proliferation of surface epithelium cells which either invade or are trapped within the cortex during ovulation (Hamilton, 1992, Radisavljevic, 1976). It has been postulated that oestrogens then promote proliferation and increase the possibility of malignant transformation (Cramer and Welch, 1983b).

Recent experimental evidence supports Fathalla's theory. Surface epithelial cells isolated from rat ovary were subjected to prolonged cell division *in vitro* by repeated subculturing. Cells acquired multiple features associated with the transformed phenotype including ability for substrate independent growth, loss of contact inhibition and the ability to form ovarian serous tumours when injected into nude athymic mice (Godwin *et al.*, 1993).

Early animal studies directly implicated hormones in ovarian malignancy. Chronic administration of oestrogens, progestins and androgens have resulted in ovarian tumours (Jabara, 1962, Gardner, 1958, Horning, 1959, Biskind and Biskind, 1944). Additionally, exposure to diethylstibestrol (DES) can induce cystadenocarcinoma *in utero* (Walker, 1984). In humans, a two-three fold excess of ovarian carcinoma has been found with women who were exposed to multiple ovulations as a result of treatment with fertility drugs (Whittemore, 1993).

The main aim of this thesis is to investigate the role of oestrogen in the growth regulation of ovarian cancer, so the actions of this hormone are discussed in further detail.

1.3.2 Steroid hormones

(i) Chemistry and biosynthesis

Steroids are naturally occuring lipids with a common ring structure consisting of three six-membered rings plus one 5-membered ring. They are synthesised mainly by the adrenal glands, testis and ovary from the precursor cholesterol which is derived mainly from low density lipoprotein (LDL) in the circulation. (reviewed in Ganong, 1987). This is summarised in Fig 1.1.

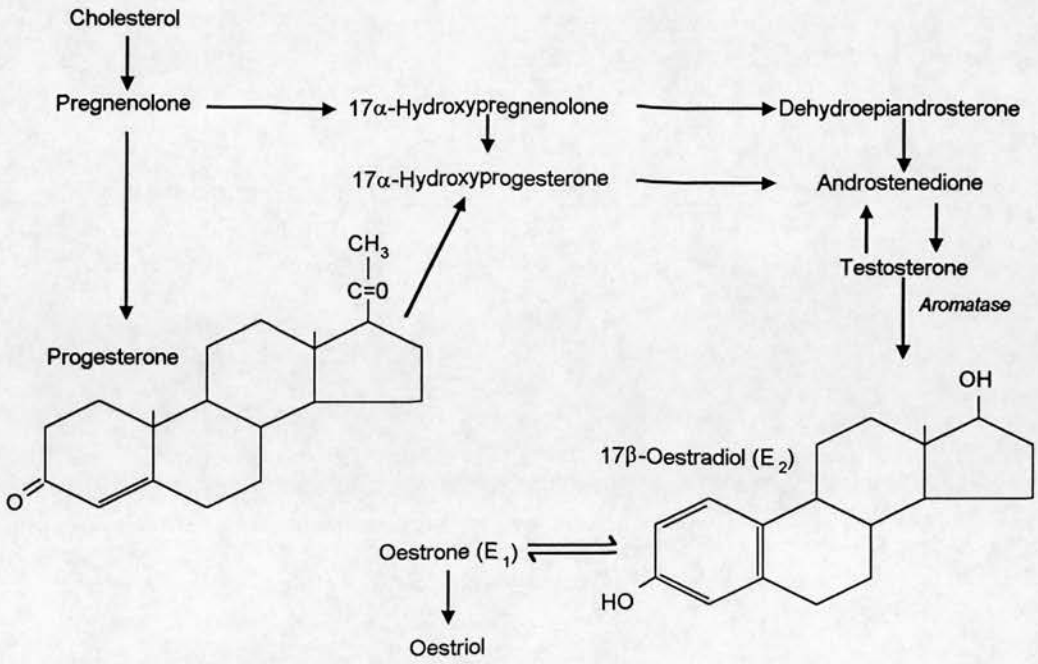


Figure 1.1

Biosynthesis of steroid hormones (Adapted from Ganong, 1989)

The two main functions of the ovary are (i) to produce ova and (ii) to synthesize three categories of steroid hormones; oestrogens, progesterone, and androgens. Oestrogens are secreted by the theca interna and granulosa cells of the ovarian follicle and by the corpus luteum. In addition, the placenta secretes oestrogen and some small quantity is also produced by the adrenal glands. The corpus luteum and placenta also secrete progesterone. The production of ovarian steroids is under the control of two hormones produced by the anterior pituitary gland; follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These in turn are under the control of leuteinizing hormone releasing hormone (LHRH) produced by the hypothalmus.

Oestrogen and progesterone levels influence these control mechanisms by a series of complex positive and negative feedback pathways (Fig 1.2).

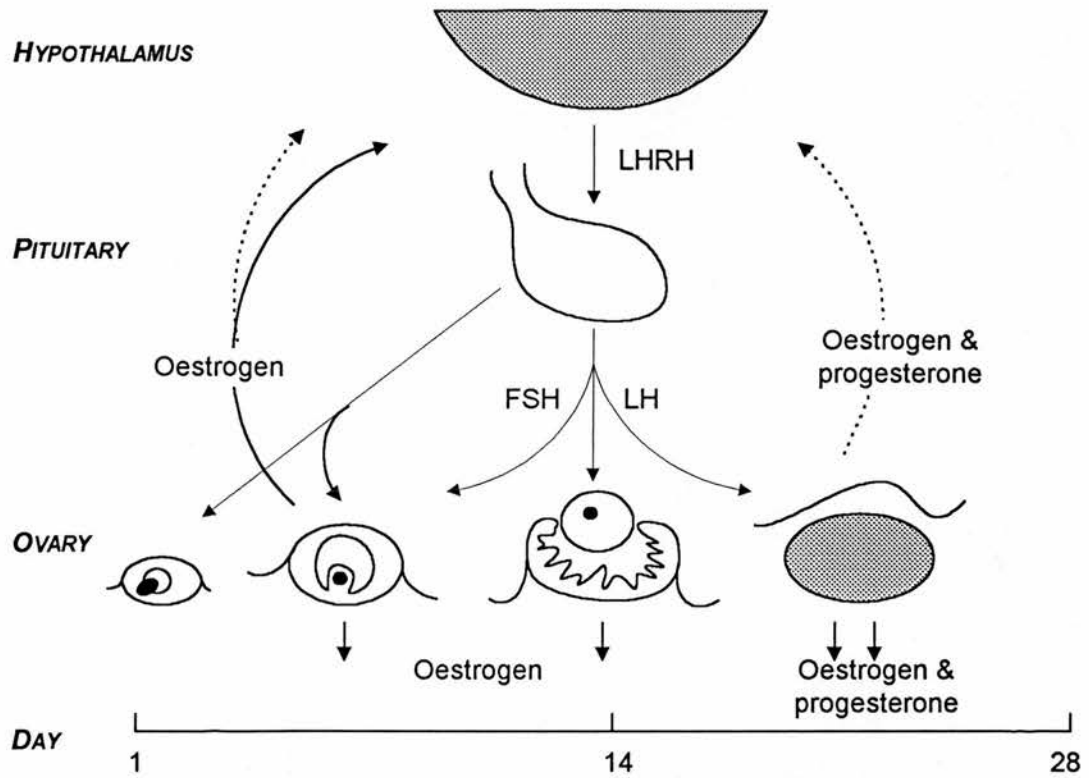


Figure 1.2

Summary of positive and negative feedback mechanisms in the control of normal ovarian steroid secretion during the menstrual cycle. Solid lines represent stimulatory effects and dashed lines inhibitory effects.

The major secreted oestrogen is 17 β-oestradiol (E_2) which is in equilibrium with oestrone (E_1) in the circulation. Oestrone in turn is metabolised to oestriol which is the least potent of the three. There are two peaks of oestrogen secretion during the

menstrual cycle; one before ovulation and the other during the mid-luteal phase. After the menopause oestrogen secretion declines to low levels. The main effects of oestrogen are to facilitate growth of ovarian follicles, increase motility of uterine tubes and increase uterine blood flow. They also produce duct growth in the breasts and have a multiple role in development of female secondary sex characteristics during puberty. Progesterone is responsible for cyclical changes in the cervix and vagina. It also decreases the number of oestrogen receptors in the endometrium and increases the conversion of 17 β-oestradiol to other less potent oestrogens.

(ii) Mechanisms of action

Oestogen exerts its major effects through interaction with a specific oestrogen receptor (ER) which resides mainly in the nucleus (King 1986, 1987a, Welshons and Gorski, 1986). There is considerable data concerning the structure of the ER. The human ER gene was originally cloned from cDNA libraries prepared from the MCF-7 breast cancer cell line (Green and Chambon, 1986, Greene *et al.*, 1986). The resultant ER protein has a molecular weight of 66kDa and is composed of 595 amino acids.

The protein consists of several functional domains which have been elucidated from DNA sequencing data used to predict amino acid composition (Figure 1.3).

The hydrophobic steroid-binding region is situated at the carboxy-terminal end of the protein and is separated from the hydrophilic DNA binding domain by a 'hinge region', the physiological relevance of which is not understood. The DNA-binding region which is rich in cysteine residues is thought to attach to chromatin DNA via

'zinc finger' projections from the protein. These are loops created by the co-ordination of a zinc ion with four of the cysteine residues (Kumar *et al.*, 1987).

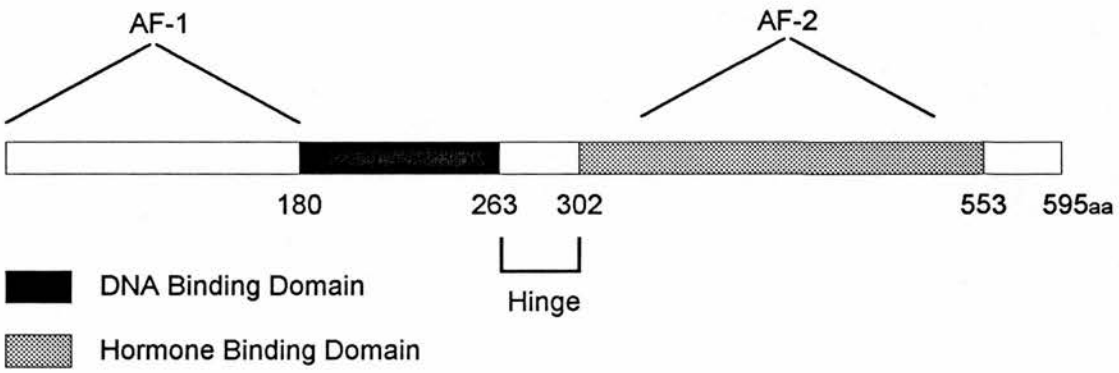


Figure 1.3

Structure of the human oestrogen receptor (Adapted from Ali *et al.*, 1993)

Although homologies are high between the steroid and DNA-binding regions of different steroid receptors, there are important differences which determine specificity of function. Genetically engineered receptors which contain the steroid binding domain of ER and the DNA binding domain of the glucocorticoid receptor (GR) will evoke a glucocorticoid-like response in the presence of oestradiol (Green and Chambon, 1987). The third functional domain of the ER is located at the amino terminus and is involved in transcriptional activation.

The unoccupied ER has a sedimentation constant of 8S which changes primarily to a 5S nuclear form after oestrogen binding (reviewed by King, 1989). This 5S form is

generated by the dimerisation of two 4S ligand binding units. The 8S form is an oligomeric complex consisting of the 68kDa oestradiol binding unit plus other proteins including the 90kDa heat shock protein, HSP90. It is thought that HSP90 may perform a role in preventing the unoccupied receptor binding to its target genes. This observation comes from evidence that after dimerisation the affinity of the DNA binding domain increases subsequent to the loss of the HSP90 protein (Chaumbraud *et al.*, 1990).

The oestrogen receptor complex binds to DNA at a site 5' to the structural gene. This region contains cis-acting enhancer sequences termed oestrogen-response elements (ERE's) which are highly specific for ER. Two regions of the ER termed transactivating functions (AF's, formerly TAF's) are able to stimulate gene transcription. AF-1, located in the N-terminal region, is able to stimulate transcription constitutively (Kumar *et al.*, 1987, Webster *et al.*, 1988, Tora *et al.*, 1989) whilst AF-2, located in the hormone binding domain requires the binding of oestrogens for its activity (Kumar *et al.*, 1986, 1987, Tora *et al.*, 1989, Webster *et al.*, 1988). AF-1 and AF-2 can both induce transcription independently in a promoter and target cell specific manner.

Binding of the receptor to DNA alone is insufficient to activate transcription. This is indicated from observations that tamoxifen-receptor complexes and unliganded receptor can bind to ERE's without activating them (Webster *et al.*, 1988). Phosphorylation of multiple serine residues is required for optimal transcription. Experiments in which serine 118 in the N-terminal region of the human ER was

mutated to an alanine residue caused a significant reduction in transcriptional activation, but DNA binding properties and nuclear binding were not altered (Ali *et al.*, 1993).

Anti-oestrogens such as 4-hydroxytamoxifen (OHT) and ICI 164,384 antagonize oestrogenic effects by competing for receptor binding. However, OHT and other tamoxifen derivatives have partial agonist activity in that although they cannot activate AF-2 they allow the ER to bind to ERE's and transcription to commence through activation by AF-1 (Berry *et al.*, 1990). Recent studies to explain this (Tzukerman *et al.*, 1994) have suggested that AF-2 may act as a transcriptional facilitator in that it is not required with certain promoters. In this context the AF-2 function may be provided by a separate transcription factor bound to the promoter. AF-1 thus acts independently of AF-2 and this may allow tamoxifen and its derivatives to exert partial agonist activity. The ICI compound is a complete antagonist which inhibits transciptional activation by both AF-1 and AF-2 (Metzger *et al.*, 1992). Both these anti-oestrogens also induce ER phosphorylation but to a lesser degree than oestrogen (Ali *et al.*, 1993).

1.3.3 Effects of oestrogen on growth

(i) Breast cancer

Many studies have reported the effects of oestrogen in breast cancer. It is the most important endocrine influence for the development and control of this carcinoma. Studies have been facilitated by the establishment of numerous oestrogen-responsive breast cancer cell lines such as MCF-7, T-47-D and ZR-75-1 (Brooks *et al.*, 1973,

Lippman *et al.*, 1976, Engel *et al.*, 1978). Oestrogen is a growth stimulator for the first two and an absolute growth requirement for ZR-75-1 (Darbre and Daly, 1989). These cell lines possess ER and are growth stimulated by physiological doses of 17 β-oestradiol (Lippman *et al.*, 1976, Weischselbaum *et al.*, 1978, Darbre *et al.*, 1983, Berthois *et al.*, 1986, Darbre *et al.*, 1989, Godden *et al.*, 1992). This growth may be inhibited by anti-oestrogens such as tamoxifen (Lippman *et al.*, 1983, Jordan, 1984, Marth *et al.*, 1984) which are used routinely for the treatment of malignant breast disease.

(ii) Ovarian Cancer

Several ovarian carcinoma cell lines have been characterised in the literature (Sinna *et al.*, 1979, Buick *et al.*, 1985, Bénard *et al.*, 1985) but there have been few studies looking at hormonal sensitivity. Part of the reason for this is the sparsity of cell lines with moderate valid ER levels. The BG-1 ovarian carcinoma line which has a relatively low ER value of 23 fmol/mg protein was demonstrated to be growth stimulated by 17 β-oestradiol (Geisinger *et al.*, 1990, Pavlik *et al.*, 1991, Galtier-Dereure *et al.*, 1992). Another ovarian carcinoma cell line, BR, is also stimulated by oestrogen but the ER status of this line was not determined (Wimalasena *et al.*, 1993). A lack of an oestrogen growth response was initially observed in the NIH:OVCAR-3 cell line which possesses an ER value of 28 fmol/mg (Nash *et al.*, 1989a), however, a later study using this cell line demonstrated that oestrogen causes a slight increase in growth if cells are incubated initially with tamoxifen (Chien *et al.*, 1994). In addition, oestrogen caused an increase in c-*myc* mRNA expression. Neither oestrogen nor tamoxifen have any effect on the growth of

the SKOV3 ovarian adenocarcinoma cell line although it expresses ER mRNA and protein (Hua *et al.*, 1995).

1.3.4 Oestrogen-regulated proteins

(i) Introduction

As part of its growth effect in breast cancer cells, oestrogen induces many enzymes involved in nucleic acid production such as DNA polymerase and uridine and thymidine kinases (Aitken, 1983, 1985). It also stimulates the turnover of phosphatidyl inositol in MCF-7 cells (Freter,1986) and induces progesterone receptor (Horwitz and McGuire, 1978a), and other proteins such as plasminogen activator (Barkley-Butler *et al.*, 1979), pS2 (Masiakowski *et al.*, 1982), HSP27 (Ciocca *et al.*, 1983) and cathepsin-D (Westley and Rochefort, 1980). Recently, oestrogen has been demonstrated to induce retinoic acid receptor-α mRNA, another member of the steroid hormone receptor superfamily (Roman *et al.*, 1993, Rishi *et al.*, 1995).

Some of these oestrogen-regulated responses are discussed in further detail below, with reference to their role in both breast and ovarian cancer.

(ii) Steroid Receptors

Oestrogen receptors can be detected in approximately 60-70% of breast tumours (McGuire, 1978) and are an important parameter in determining the hormonal sensitivity of the tumour and its likely response to endocrine therapy. The progesterone receptor is one of a number of proteins induced by oestrogen (Horwitz and McGuire,1978a) and assessment of both the ER and PR status of breast tumours may improve the clinical management of the disease. Patients with both ER and PR

show a good response to endocrine treatment, whilst those lacking both receptors gain little benefit (Clark and McGuire, 1989).

The presence of oestrogen receptors (ER) in ovarian cancers also implicates oestrogen in this disease, and progesterone receptors may be indicative of oestrogen sensitivity. Oestrogen receptors are found at low levels in normal human ovarian surface epithelium with progesterone receptors being more prevalent (reviewed in Leake and Owens, 1990). However in ovarian cancer, incidence of PR is generally lower whereas ER increases (Quinn *et al.*, 1982, Spona *et al.*, 1983). A review of 52 reports by Slotman and Rao, (1988) revealed that ER were present on 63% of ovarian tumours, PR on 48% and ER and PR together on 34% of the tumours. Neither were detected in 25% of cases. In addition androgen receptors were present on 69% of tumours. A relationship between endometrioid tumours and PR status has been reported, often in conjunction with ER (Freidman *et al.*, 1979, Ford *et al.*, 1983, Sutton *et al.*, 1986). Serous tumours are often ER-positive (Quinn *et al.*, 1982) whilst mucinous and clear cell tumours possess lower amounts of steroid receptors (Quinn *et al.*, 1982, Ford *et al.*, 1983).

As in breast cancer, ER presence in ovarian cancer has been linked with a good prognosis (Creasman *et al.*, 1981, Kauppila *et al.*, 1983, Bizzi *et al.*, 1988, Kieback *et al.*, 1993a,b) although others have not found any correlation (Sevelda *et al.*, 1990, Rose *et al.*, 1990). Inconsistencies in results may be due to differences in methodologies or cut-off values, and recently studies have used the CARL value (**C**omposition **A**djusted **R**eceptor **L**evel) which takes into account the cellular

heterogeneity within tumours (Kieback *et al.*, 1993a), or obtained an immunoreactive score comprising both the percentage of cell staining positively for ER and the staining intensity (Kieback *et al.*, 1993b). Both these methods have shown an asssociation between ER positivity and improved survival (Kieback *et al.*, 1993a,b). A similar relationship between PR positivity and survival has been reported (Iversen *et al.*, 1986, Sevelda *et al.*, 1990, Harding *et al.*, 1990, Kommoss *et al.*, 1992a).

As mentioned previously, several ovarian cancer cell lines possessing low levels of ER have been described which vary in their growth responses to oestrogen (Nash *et al.*, 1989a, Galtier-Dereure *et al.*, 1992, Chien *et al.*, 1994). Oestrogen induction of progesterone receptors has been demonstrated in the NIH:OVCAR-3 ovarian cancer cell line (Nash *et al.*, 1989a) and *in vivo* (Hamilton *et al.*, 1984).

(iii) pS2 (pNR-2)

a) Structure and function

The pS2 protein (also known as pNR-2) is one of a series of oestrogen-inducible proteins and has been cited as a possible predictive marker of endocrine response in human breast carcinomas. pS2 was first characterised in the breast cancer cell line MCF-7 from cDNA sequences (Masiakowski *et al.*, 1982) corresponding to an mRNA which was specifically increased by oestrogen treatment. Cloning of the gene revealed that the full non-polyadenylated transcript is 490 nucleotides in length and organised into three exons (Jeltsch *et al.*, 1987) which encode a small pre-protein of 84 amino acids (Jackowlev *et al.*, 1984), leading to a mature secreted protein of 60 amino acids (Rio *et al.*, 1988a). The gene has been mapped to chromosome 21q22.3

(Moisan *et al.*, 1988). Berry *et al.*, (1989) demonstrated that when isolated, the transcriptional enhancer oestrogen-responsive element of the gene can confer oestrogen inducibility to the rabbit β-globin gene promoter, an action which is inibited by anti-oestrogens. Further studies have also revealed that enhancer regions flanking the 5' region of the gene are responsive to TPA, EGF and the c-H-*ras* and c-*jun* proto-oncogenes (Nunez *et al.*, 1989).

The function of the pS2 protein has yet to be elucidated but it shows some structural similarities to small peptide growth factors such as human IGF-I and IGF-II. There is also a high degree of homology between the pS2 protein and a group of peptides which include the porcine spasmolytic polypeptide, PSP and spasmolysin, a repetitive protein isolated from *Xenopus* skin (reviewed in Rio and Chambon, 1990).

b) Distribution

A wide screen by immunohistochemistry of normal tissues including colon, pancreas, liver, lung, prostate, kidney, endometrium, ovary and pituitary gland detected no expression of pS2 protein (Rio *et al.*, 1988b). However the protein has been detected in normal gastric mucosa, small intestinal mucosa and breast epithelium (Rio *et al.* 1988b, Piggott *et al.* 1991), and in a variety of malignant tissues including gastric carcinomas, breast and gynaecological tumours (Luqmani *et al.*, 1989, Rio *et al.*, 1987, Wysocki *et al.*, 1990, Henry *et al.*, 1991b).

c) Prognostic value

pS2 expression correlates predominantly with ER-positive breast tumours (Rio *et al.*, 1987, Foekens *et al.*, 1990a, Henry *et al.*, 1991a, Schwartz *et al.*, 1991, Thor *et al.*,

1992, Stonelake *et al.*, 1994). It has also been associated with later recurrence and death when analysed by immunoradiometric assay (Foekens *et al.*, 1990a), with overall survival being greatest in ER/PR/pS2-positive tumours. However, other groups using immunohistochemistry found no association between pS2 expression and overall survival or time to first relapse (Henry *et al.*, 1991a, Thor *et al.*, 1992, Dookeran *et al.*, 1993).

Using immunohistochemistry, Henry *et al.*, (1991b) detected pS2 expression in benign and malignant ovarian epithelial tumours, with a strong correlation with the mucinous subtype. Wysocki *et al.*, (1990) also demonstrated pS2 mRNA in ovarian carcinomas and noted a correlation with ER status although this was not statistically proven. However Foekens *et al.*, (1990b) found no expresssion of pS2 in ovarian tumours using a radiometric immunoassay.

(iv) HSP27

a) Structure and function

HSP27 is a 27kDa member of the heat shock protein (HSP) family which is induced in response to oestrogen and has been cited as a possible prognostic marker in breast cancer. It was first reported as a 24,000 molecular weight protein, 'p24', which was induced in MCF-7 breast cancer cells in response to oestrogen (Edwards *et al.*, 1980). It was also induced by heat shock in this cell line (Fuqua *et al.*, 1989). Several other investigators were also simultaneously looking at the protein. Thor *et al.*, (1991) described srp27 expression in human breast carcinomas and Coffer and King, (1988) detected a cytoplasmic phosphoprotein, 'p29' with an antibody, D5, raised

against partially purified human myometrial ER. Using immunocytochemical and immunoblot techniques, Ciocca and Luque, (1991) identified p29 as HSP27.

The human HSP27 gene has been characterised (Hickey *et al.*, 1986). From this and other studies it was noted that HSP27, like other small HSP's, shares a striking homology with α-crystallin lens proteins. HSP gene regulation has been well documented and involves two heat shock factors, HSF1 and HSF2 in humans (Rabindran *et al.*, 1991, Schuetz *et al.*, 1991). Heat shock-induced transcription is activated by binding of HSF(s) to the heat shock element (HSE) of the HSP gene. It is thought that hormonal activation of HSP's may be similar to a system described in *Drosophila* whereby β-ecdysone binds to a distinct promoter region, different to the heat shock element. The β-ecdysone response element shows similarities to the mammalian oestrogen response element (Riddihough and Pelham, 1987).

In addition to being induced by oestrogen, HSP27, as its name suggests, is induced by heat shock and other environmental stresses such as viral or microbial infections, exposure to oxidants and heavy metals (reviewed in Ciocca *et al.*, 1993). Besides its role in thermotolerance, HSP27 is also thought to act as a molecular chaperone and play a role in signal transduction. In addition, recent studies have implicated it in the development of drug resistance in a number of cell types (Huot *et al.*, 1992) including breast cancer cell lines (Ciocca *et al.*, 1992, Oesterreich *et al.*, 1993).

b) Distribution

The heat shock family are found in nearly all living cells, both eukaryote and prokaryote. In human tissues, HSP27 has been detected in muscle, nervous and

connective tissue (Ciocca *et al.*, 1983). It is expressed in higher amounts in oestrogen target organs of the female reproductive tract, including the uterus, vagina and fallopian tubes (Ciocca *et al.*, 1983, reviewed in Ciocca *et al.*, 1993).

c) Prognostic value

It is unclear how HSP27 may predict for prognosis in breast cancer. There has been conflicting data regarding its utility in this disease. Certain groups have reported an association with more aggressive breast tumours (Chamness *et al.*, 1988, Tandon *et al.* 1990) and a short disease free survival (Thor *et al.*, 1991, Love and King, 1994). However Seymour *et al.*, (1990) reported an improved outcome in HSP27 positive tumours. There have been no reports of its presence in ovarian cancer.

(v) Cathepsin D

Cathepsin D was first described in the oestrogen-receptor positive breast cell lines MCF-7 and ZR-75-1 where it was secreted as a 52kDa protein into the culture medium after oestrogen treatment (Westley and Rochefort, 1980). The 52kDa protein was later purified and shown to be a precursor, approximately 55% of which is secreted while the rest is processed into a 48kDa form and a 34kDa plus 14kDa protein. The exact function of this protein is unknown but evidence points to it playing a role in mammary carcinogenesis perhaps by enhancing tumour invasion (Rochefort *et al.*, 1987). Purified Cathepsin D is able to stimulate oestrogen-deprived MCF-7 cells by activation of latent forms of growth factors, and at acidic pH can degrade the extracellular matrix, suggesting it may mediate the effects of oestrogens on tumour growth and invasion.

Cathepsin D has been detected in breast biopsies and has been demonstrated to be independent of steroid receptor status (Cavailles *et al.*, 1987). It is constitutively overexpressed in ER-negative breast cells (Rochefort *et al.*, 1990) and linked in a number of studies to increased risk of metastasis (Thorpe *et al.*, 1989, Spyratos *et al.*, 1989, Tandon *et al.*, 1990b). Recent studies have demonstrated its modulation by oestrogen in several ovarian cancer cell lines (Galtier-Dereure *et al.*, 1992, Hua *et al.*, 1995). Production of this protein by some ER-negative cells (Rochefort, 1994) suggests that it will not be a useful marker of hormonal sensitivity in ovarian cancer. Cathepsin D has been detected in some ovarian tumour cytosols and higher levels have been observed in omental metastases than primary tumours but its presence analogous to the situation found in breast cancer does not correlate with ER or PR. (Scambia *et al.*, 1991, 1994).

1.3.5 Growth factors

(i) Introduction

Growth factors are polypeptides that in a normal context act as extracellular agents to modulate cell function or induce quiescent cells to proliferate or differentiate (Roberts and Sporn, 1989). Smith *et al.*, (1971) proposed that growth factors cancel certain restriction points within the cell cycle and allow it to progress. In human cancer the expression and function of peptide growth factors and/or their receptors are frequently deranged leading to the overexpression of normal or mutated forms and resulting in the development of malignancies (Roberts and Sporn, 1989, Dickson, 1990). Growth factors exert their biological effects on target cells following interaction with specific receptors which then trigger a series of enzymatic and

biological events, ultimately leading to DNA synthesis and effects on cellular kinetics. They may act on the same cell which secreted them (autocrine regulation), or on neighbouring cells (paracrine regulation) or on distant cells (endocrine regulation) (see Figure 1.4).

There is an increasing amount of evidence to suggest that autocrine mechanisms are important in the growth control of ovarian cancer. The major autocrine influences are probably effected though members of the epidermal growth factor/transforming growth factor-alpha (EGF/TGF-α), insulin-like growth factor (IGF) and TGF-β families (Kohler *et al.*, 1989, Owens *et al.*, 1991a, Stromberg *et al.*, 1992, Hurteau *et al.*, 1994). Data from breast cancer cell lines indicates that oestrogen may mediate its mitogenic effects through growth factors which include TGF-α and IGF-I (Dickson *et al.*, 1986, Bates *et al.*, 1988, Huff *et al.*, 1988). Several reports also suggest this may be true in ovarian cancer; both TGF-α mRNA and several IGF binding proteins are modulated by oestrogen (Nash *et al.*, 1989b, Krywicki *et al.*, 1993).

The role of oestrogen and its effects on EGF, TGF-α, and IGF-I receptor expression in ovarian cancer cell lines is examined in this thesis, and so these growth factor families are discussed in further detail.

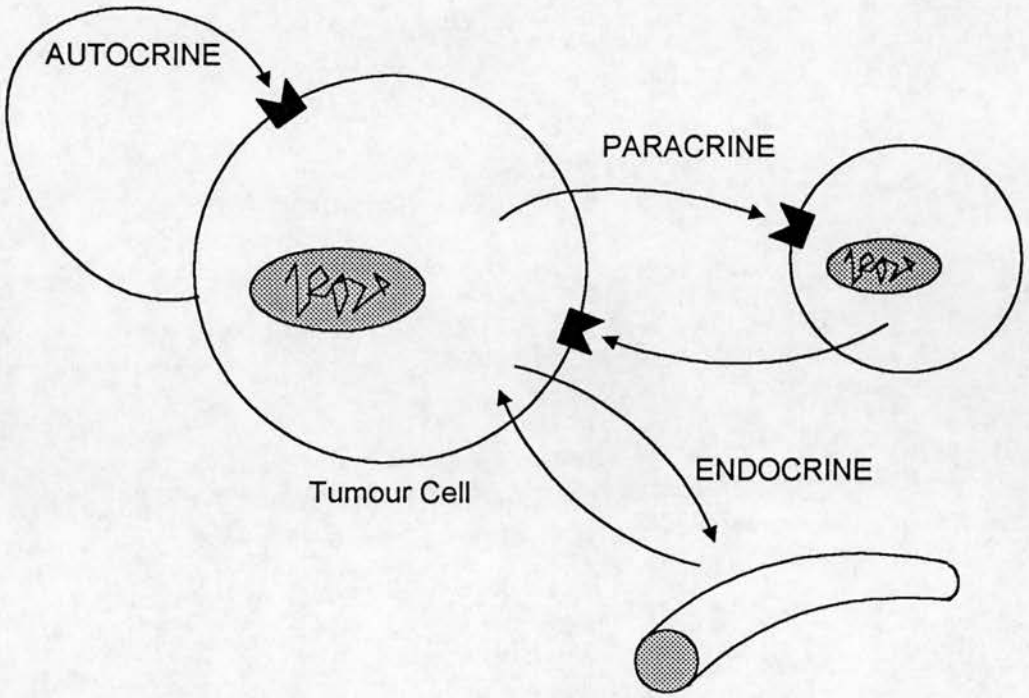


Figure 1.4

Possible growth regulation pathways

(ii) Structure and synthesis of Epidermal Growth Factor (EGF)

Epidermal growth factor was first discovered by Stanley Cohen over thirty years ago as a contaminant of nerve growth factor (1962). It was originally distinguished for its ability to open the eyelids of newborn mice and accelerate the eruption of mouse teeth, and was found in abundance in mouse salivary glands. This source enabled the purification and elucidation of its amino acid structure (reviewed in Burgess, 1989). Gregory, (1975) realised that murine EGF was very similar in structure to a human urinary protein responsible for the inhibition of gastric acid secretion, β-urogastrone, which his group had recently purified. Both factors have 37 amino acids (out of 53) in

common and each contains 3 disulphide bonds at similar positions. He concluded that human EGF and urogastrone were 'one and the same'.

EGF is synthesised as a 1217 amino acid precursor which is modified to a mature 53 amino acid form with a molecular weight of 6045 (Gray *et al.*, 1983, Scott *et al.*, 1983). Originally thought to have limited expression it was found from sections of whole newborn mice that EGF is expressed in a wide variety of tissues (Rall *et al.*, 1985) although the only concentrated source in the mouse is the male submaxillary glands (Byyny *et al.*, 1972). Here the precursor is broken down to the 53 amino acid mature form which associates with several binding proteins in cytoplasmic granules (Kasselberg *et al.*, 1985).

EGF has many varied biological actions both *in vivo* and *in vitro.* In particular it has proliferative effects on fibroblasts, keratinocytes and epithelial cells (reviewed in Burgess, 1989). In humans, EGF is found in a variety of fluids including breast milk, colostrum and urine (Jaspar and Frankchimont, 1985) and is located in a wide variety of epithelial cells including those from lung, stomach, kidney, pancreas, skin, breast, ovary, uterus and placenta (reviewed in Modjtahedi and Dean, 1994). It has been shown to promote the proliferation of human mammary epithelial cells in culture (Ethier *et al.*, 1991) as well as a number of other normal and tumour cells.

(iii) Structure and synthesis of Transforming Growth Factor-alpha (TGF-α)

Transforming growth factors were originally discovered by De Larco and Todaro, (1978) in the conditioned media of murine sarcoma virus-transformed mouse 3T3

fibroblasts and provided evidence that expression of a viral oncogene could turn on production of a cellular growth factor. The 'factor' involved was initially called sarcoma growth factor (SGF) and had the ability to cause a phenotypic transformation of cells; conditioned medium from the transformed cells induced a reversible change in the morphology of normal fibroblasts (NRK cells) to a transformed appearance, caused loss of contact inhibition of cells in monolayer culture and induced anchorage-independent colony formation in soft agar (De Larco and Todaro, 1978, Roberts *et al.*, 1983). This data provided the first evidence for the autocrine secretion of growth factors being a causative factor in malignant transformation (Sporn and Todaro, 1980). When SGF was purified this transforming activity was found to be the result of two growth factors, TGF-α and TGF-β. Originally thought to be a property of transformed cells, it was discovered that these two growth factors could also be extracted from normal cells (Roberts *et al.*, 1981, Sporn *et al.*, 1986).

Human TGF-α cDNA was first isolated and characterised from a library prepared from a human renal carcinoma cell line (Derynck *et al.*, 1984). It was found that the mRNA of approximately 4.8Kb encoded a 160 amino acid polypeptide anchored to the cell surface via its C-terminus, from which the 50 amino acid biologically active form derives (Bringman *et al.*, 1987). In addition to this, higher molecular weight forms are also released from the precursor. The portion of TGF-α attached to the cell membrane is palmitoylated and it has been suggested that this may allow for more efficient cleavage of the precursor by slowing down its passage through the golgi and cell surface membranes. Possibly the membrane-bound TGF-α molecule plays a role

in cell-cell signalling. TGF-α shows considerable structural and biological homology with EGF and binds to the EGF receptor with similar affinity (Todaro *et al.*, 1980, Carpenter *et al.*, 1983). TGF-α may play an important role in foetal development and tissue regeneration (Derynck, 1992). Moreover, many studies also indicate that human cancer cells produce and release TGF-α, and possess functional receptors for the peptide (Todaro *et al.*, 1980, Roberts *et al.*, 1980, reviewed in Modjtahedi and Dean, 1994).

(iv) Structure of the Epidermal Growth Factor Receptor

The EGF receptor (EGFR) is possibly one of the most studied tyrosine kinase receptors and is found in a wide variety of tissues and cell types with the exception of haemopoietic cells. It belongs to the Type I tyrosine kinase growth factor receptor family which includes the products of the c-*erb*B-2 (also known as *neu*/*HER*-2), c-*erb*B-3 and c-*erb*B-4 proto-oncogenes (Coussens *et al.*, 1985, Yamamoto *et al.*, 1986, Kraus *et al.*, 1989, Plowman *et al.*, 1993). The gene for the EGFR, c-*erb*B-1 is located on chromosome 7 (reviewed in Hsuan *et al.*, 1989). This name derives from the original sequencing experiments of the cloned receptor which revealed a high level of sequence homology with the *erb*-B oncogene of the avian erythroblastosis virus (AEV) (Ullrich *et al.*, 1984). It is overexpressed in many malignancies including some lung cancers, gastric carcinomas and breast cancer (Gullick *et al.*, 1991). It was first cloned and sequenced in 1984 (Downward *et al.*, 1984, Ullrich *et al.*, 1984). The protein consists of a single polypeptide chain of 1186 amino acids, divided into two domains separated by a hydrophobic membrane-spanning region. During its biosynthesis N-linked oligosaccharide chains are added co-translationally to a 135kDa

polypeptide core and a 24 amino acid leader sequence removed to give rise to a 160kDa precursor molecule, a process inhibited by tunicamycin. Further modification of the oligosaccharide chains yields the mature 170kDa receptor (Mayes and Waterfield, 1984). These oligosaccharide chains have been shown to confer ligand binding ability to the receptor as tunicamycin-treated cells lack this facility. The extracellular domain contains 621 amino acids and is responsible for binding the ligand. It is folded into 4 domains termed L1, L2, S1 and S2, the two L-domains being folded together to form a β-barrel superstructure which binds the ligand and is supported by the S domains (Bajaj *et al.*, 1987). These two S domains contain a high cluster of cysteine residues (51 out of 621 amino acids) and are probably involved in the formation of disulphide bonds. The transmembrane region of the receptor consists of 23 uncharged hydrophobic residues and is held in its position by a highly-basic stop transfer sequence. The exact function of this region has not been determined but experiments in which the lipid environment of cell lysates is modifed can alter both ligand binding and kinase activity (Downward *et al.*, 1985). The intracellular region of the receptor is responsible for the tyrosine kinase activity and consists of about 250 amino acids leading from a 50 amino acid juxta-membrane sequence. The catalytic domain is linked to an autophosphorylation domain of about 150-200 amino acids.

After binding, receptors dimerize and rapidly become phosphorylated on tyrosine residues in the C-terminal autophosphorylation domain. Recent work implicates growth factor receptor-binding protein 2 (GRB2) as a primary signal transduction protein involved in EGFR siganalling (Rozakis-Adcock *et al.*, 1993, Egan *et al.*, 1993). GRB2, in a heterodimer complex with mSOS protein, binds to the

phosphorylated EGFR; this complex then binds the ras-GDP complex (Chardin *et al.*, 1993). This leads to the activation of raf kinase probably through the binding of c-raf-1 proto-oncogene (Zhang *et al.*, 1993, Warne *et al.*, 1993) and serves to activate the MAP kinase cascade (Kyriakis *et al.*, 1992). Targets for MAP kinase include nuclear transcription factors c-*myc* and c-*jun* (Alavrez *et al.*, 1991, Pulverer *et al.*, 1991) and these in turn influence cell cycle regulation. As well as acting as a receptor for EGF and its related polypeptide TGF-α, the EGFR can also bind several virally encoded growth factors such as those produced by *Vaccinia* (Brown *et al.*, 1985, Stroobant *et al.*, 1985), and amphiregulin, a growth factor first identified in breast cancer conditioned medium (Shoyab *et al.*, 1988, 1989). More recently a new epidermal growth factor-related protein has been described (Ciccodicola *et al.*, (1989). Cripto-1 (CR-1) is a 188 amino acid protein which shares a cysteine rich motif in common with the other members of the EGF family. It is overexpressed in many colorectal and gastric carcinomas (Ciardiello *et al.*, 1991, Kuniyasu *et al.*, 1991), and in pancreatic cancer (Freiss *et al.*, 1994). However, recent work suggests that it functions as a growth factor in an EGFR-independent pathway (Brandt *et al.*, 1994).

(v) Implication of the EGF/TGF-α family in ovarian cancer

Evidence is accumulating for growth factor involvement in the proliferation of ovarian cancer. The presence of EGFR have been detected by both ligand binding and immunohistochemistry in between 33 and 75% of ovarian cancers (Bauknecht *et al.*, 1989, Morishige *et al.*, 1991a, Owens *et al.*, 1991b, Jindal *et al.*, 1994) and appear to be higher in malignant cells as compared to benign or normal ovary, implying a role

for EGFR in carcinogenesis (Owens and Leake, 1993, Berns *et al.*, 1992). TGF-α has been reported to be present in 50-100% of ovarian tumours (Owens *et al.* 1991a, Morishige *et al.*, 1991, Kommoss *et al.*, 1990b). EGF has also been confirmed in between 28-71% of ovarian tumour samples (Owens *et al.*, 1991a, Stromberg *et al.*, 1994) and the presence of amphiregulin and cripto has also been reported (Stromberg *et al.*, 1994). The majority of studies report that the presence of EGFR is associated with poor prognosis in malignant ovarian tumours, (Bauknecht *et al.*, 1989, Battaglia *et al.*, 1989, Berchuck *et al.*, 1991, Scambia *et al.*, 1992) although this is not universally observed (Van der Burg *et al.*, 1993, Bauknecht *et al.*, 1990).

C-*erb*B-2 is overexpressed in about 20-30% of ovarian tumours and is associated with poor prognosis, similar to EGFR (Slamon *et al.*, 1989, Berchuck *et al.*, 1990a). A recent study also reported the presence of the c-erbB-3 receptor in 89% of malignant ovarian tumours, with a strong correlation between overexpression and borderline and early invasive tumours (Simpson *et al.*, 1995).

Several studies have demonstrated the presence of EGFR and TGF-α production in a range of ovarian carcinoma cell lines, and observations that neutralising antibodies to TGF-α inhibit proliferation support the view of an EGFR/TGF-α autocrine loop in ovarian cancer (Morishige *et al.*, 1991b, Stromberg *et al.*, 1992, Jindal *et al.*, 1994). A similar finding was noticed with an ovarian cell line developed *in vivo* (Kurachi *et al.*, 1991), and in primary human adenocarcinomas (Morishige *et al.*, 1991a). Berchuck *et al.*, (1990b) demonstrated that addition of epidermal growth factor (EGF) induced growth in several ovarian cancer cell lines but transforming growth

factor-beta (TGF-β) caused an inhibition. Most of the cell lines were also found to produce TGF-β, but not EGF. Bartlett *et al.*, (1991, 1992) have demonstrated varying expression of both TGF-α and TGF-β mRNA's in the series of ovarian lines described in this thesis. Three of these same cell lines possess EGFR and are growth stimulated in response to exogenous EGF and TGF-α (Crew *et al.*, 1992a). In one of these ER-positive ovarian cancer cell lines, PEO4, oestrogen has been shown to increase TGF-α mRNA levels, (Nash *et al.*, 1989) and to downregulate levels of EGFR (Crew *et al.*, 1992b). There is also evidence that oestrogen may modulate TGF-α *in vivo*, from data showing that higher levels of TGF-α are found in ER-positive/PR-positive ovarian tumours (Leake *et al.*, 1995).

(vi) Insulin-Like Growth Factors

The insulin-like growth factors IGF-I and II are low molecular weight peptides which are structurally similar to pro-insulin and are believed to play a role in cyclic ovarian follicular development (Giordano *et al.*, 1992, reviewed in Giudice, 1992). The IGF-I receptor is the main mediator of IGF activity although IGF's can also act through the insulin receptor. The growth stimulatory potential of IGF's is modulated by binding proteins (IGFBP's) which may target them to specific tissues (Jones and Clemmons, 1995). Alternatively IGFBP's may inhibit the effects of IGF's by complexing with them. They have been implicated in the autocrine regulation of a number of tumour cell lines (Macaulay, 1992), and evidence is accumulating to suggest that oestrogen may exerts part of its actions through modulation of IGF's. In the ER-positive breast cancer cell line MCF-7, oestrogen has been shown to upregulate the IGF-I receptor

(Stewart *et al.*, 1990), and Brünner *et al.*, (1993b) showed that oestrogen treatment of human breast tumour xenografts grown in nude mice correlated with IGF-II expression. IGF-I, and its receptor are expressed in several ovarian carcinoma cell lines (Yee *et al.*, 1991a) and Resnicoff *et al.*, (1993) have shown that growth of the OVCAR-3 and CaOV-3 ovarian carcinoma cell lines is stimulated by IGF-I. Furthermore, antisense oligonucleotides against the IGF-I receptor inhibit proliferation of OVCAR-3. A number of IGFBP's (IGFBP2 to IGFBP-6) are also expressed by ovarian carcinoma cell lines (Yee *et al.*, 1991a, Hofmann *et al.*, 1994) and have been found in malignant ovarian cyst fluid (Kanety *et al.*, 1996). Data regarding oestrogen modulation of the IGF family in ovarian cancer is sparse, although Krywicki *et al.*, (1993) have shown that oestrogen downregulates the expression of IGFBP3, but upregulates IGFBP5 in the PEO4 ovarian cell line.

(vii) Summary

Thus, an increasing amount of evidence suggests that growth factors are implicated in the growth of ovarian cancer cells. Moreover, oestrogen may influence the growth of hormonally-sensitive ovarian cancer cells through the regulation of these factors. A diagram illustrating some of the possible response pathways through which oestrogen may mediates its effects on growth is shown in Figure 1.5

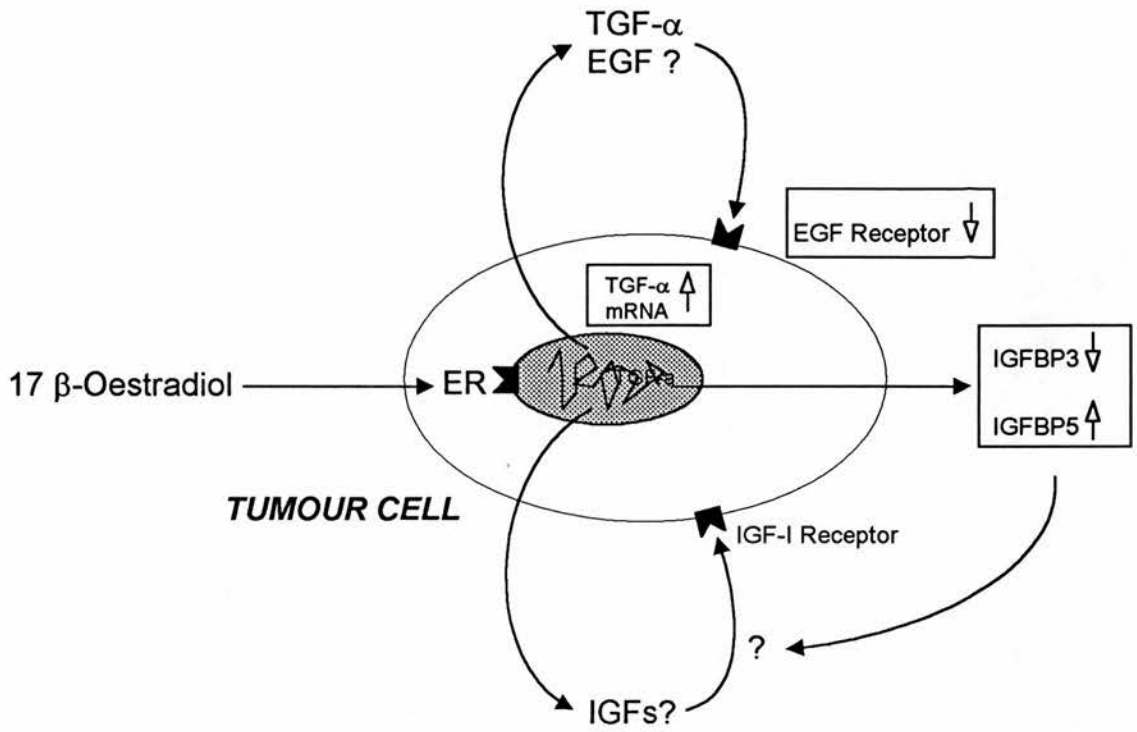


Figure 1.5

A summary diagram illustrating some of the possible mechanisms by which oestrogen may regulate growth in a hormonally sensitive ovarian cancer cell.

1.4 Aims of the study

As described in this introduction, there is both epidemiologcal and direct evidence to suggest that at least a percentage of ovarian cancer is under the control of oestrogen. The aims of this study were to obtain direct evidence of oestrogen regulation in ovarian carcinoma cells and to define further oestrogenic mechanisms of growth control. To facilitate this, a series of ovarian carcinoma cell lines established and characterised within the Edinburgh Medical Oncology Unit were employed in experiments. These ovarian cell lines possess a range of oestrogen receptors and include the first ovarian cancer cell lines described with substantial levels of ER. Using these cell lines the role of the hormone oestrogen was to be assessed by determining :

a) the effects of oestrogen on the growth of human ovarian cancer cells in culture and relating this to ER content.

b) which factors may be putative markers of hormonal sensitivity in these lines.

c) whether the effects of oestrogen on growth may be mediated through the action of growth factors.

2. MATERIALS AND METHODS

2.1 MATERIALS

The sources of materials used in the experiments discussed in this thesis are listed in the following section according to experimental technique.

2.1.1 Cell culture

(i) *In vitro* cell lines

a) Ovarian cancer cell lines

The serous adenocarcinoma ovarian cell lines were derived from ascitic fluids as previously described (Langdon *et al.*, 1988) and brief characterisation details are given in Table 2.1. The cis-platinum (CDDP) resistant subline $PEO1^{CDDP}$ was derived from the PEO1 cell line by the continuous culture of the parent line in increasing concentrations of CDDP from 5×10^{-9}M to 10^{-6}M over a six month period. The cytology and karyotype of the subline is similar to the parent line but the doubling time is shorter, plating efficiency is higher and resistance to CDDP is five-fold greater than that observed for the parental PEO1 cell line when assessed by MTT assay.

b) Breast cancer cell lines

Two breast cancer cell lines, ZR-75-1 and MDA-MB-231 were incorporated into the studies for comparison. The ZR-75-1 line is an extensively studied ER-positive breast cancer cell line first characterised by Engel *et al.*, (1978). It was derived from the malignant ascitic effusion of a 63 year old women suffering from an infiltrating duct carcinoma of the right breast. The woman at the time had received 3 months of chemotherapy including tamoxifen and fluoxymesterone. The MDA-MB-231 breast

carcinoma cell line was derived from a pleural effusion, (Cailleau *et al.*, 1974), and is ER-negative (Horwitz *et al.*,1978c).

c) Materials for *in vitro* cell culture experiments

The following materials were used in the routine culture of the *in vitro* cell lines

Cell Scrapers	Costar Corp.
Activated charcoal	Sigma
Dimethyl Sulphoxide (DMSO)	BDH Analar
Dextran T70	Pharmacia
DMEM media with/without phenol red	GIBCO Paisley, UK
EDTA	Sigma
Foetal Calf Serum	GIBCO Paisley, UK
L-glutamine	Sigma
17 β- oestradiol	Sigma
Penicillin / Streptomycin	GIBCO Paisley, UK
Phenol red	Flow Laboratories
Phosphate Buffered Saline (PBS)	Oxoid
RPMI 1640 media with/without phenol red	GIBCO Paisley, UK
Sulphatase	Sigma
Tissue culture flasks, multi-well plates	Corning, Falcon
Trypsin	GIBCO Paisley, UK

Table 2.1 Characterisation of cell lines

CELL LINE	ORIGIN OF CELL LINES INDICATING CLINICAL CHARACTERISTICS AND TREATMENT OF PRIMARY TUMOUR	PATIENT	OESTROGEN RECEPTOR STATUS (fmol/mg protein)
Ovarian cancer cell lines			*a*
PEO1	**Derived from the peritoneal ascites (PA) of a patient with a poorly differentiated serous adenocarcinoma following treatment with CDDP, 5-FU and chlorambucil**	**DB**	**96**
PEO4	**Derived from PA in the same patient following the development of drug resistance**		**112**
PEO6	**Derived from PA in the same patient prior to death**		**132**
PEO1^{CDDP}	**Derived from *in vitro* culturing of PEO1 cell line in increasing concentrations of CDDP**		**ND**
PEA1	**Derived from a pleural effusion of a patient with a poorly differentiated adenocarcinoma. No treatment**	**MK**	**23**
PEA2	**Derived from the PA from the same patient, MK after relapse from cisplatin and prednimustine therapy**		**12**
PEO16	**Derived from the PA of a patient with a poorly differentiated serous adenocarcinoma after radiotherapy**	**ER**	**0**
PEO14	**Derived from the PA of a patient with a well differentiated serous adenocarcinoma prior to treatment**	**EM**	**0**
PEO23	**Derived from the PA of the same patient, EM collected on relapse after cisplatin and chlorambucil therapy**		**0**
Breast cancer cell lines			
ZR-75-1	**Malignant ascitic effusion from patient receiving endocrine therapy**		**214** [b]
MDA-MB-231	**Pleural effusion**		**0** [c]

a ER status of routinely cultured ovarian cell lines. Values shown are the mean of three measurements determined by a ligand binding assay or sucrose density gradient assay (Langdon *et al.*, 1990) *b* Long *et al.*, (1992) *c* Horwitz *et al.*, (1978c) ND = Not determined

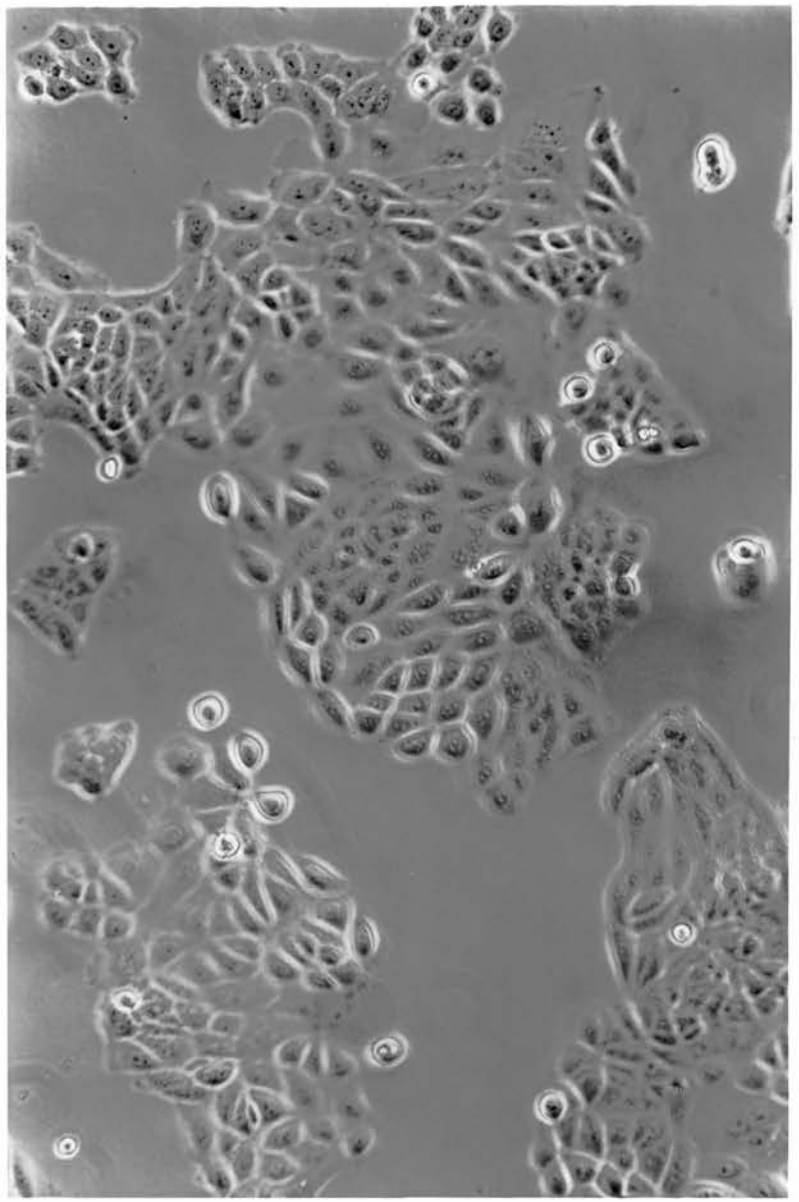


Figure 2.1

Photograph of the PEO1 ovarian carcinoma cell line in mid-log phase (x 125)

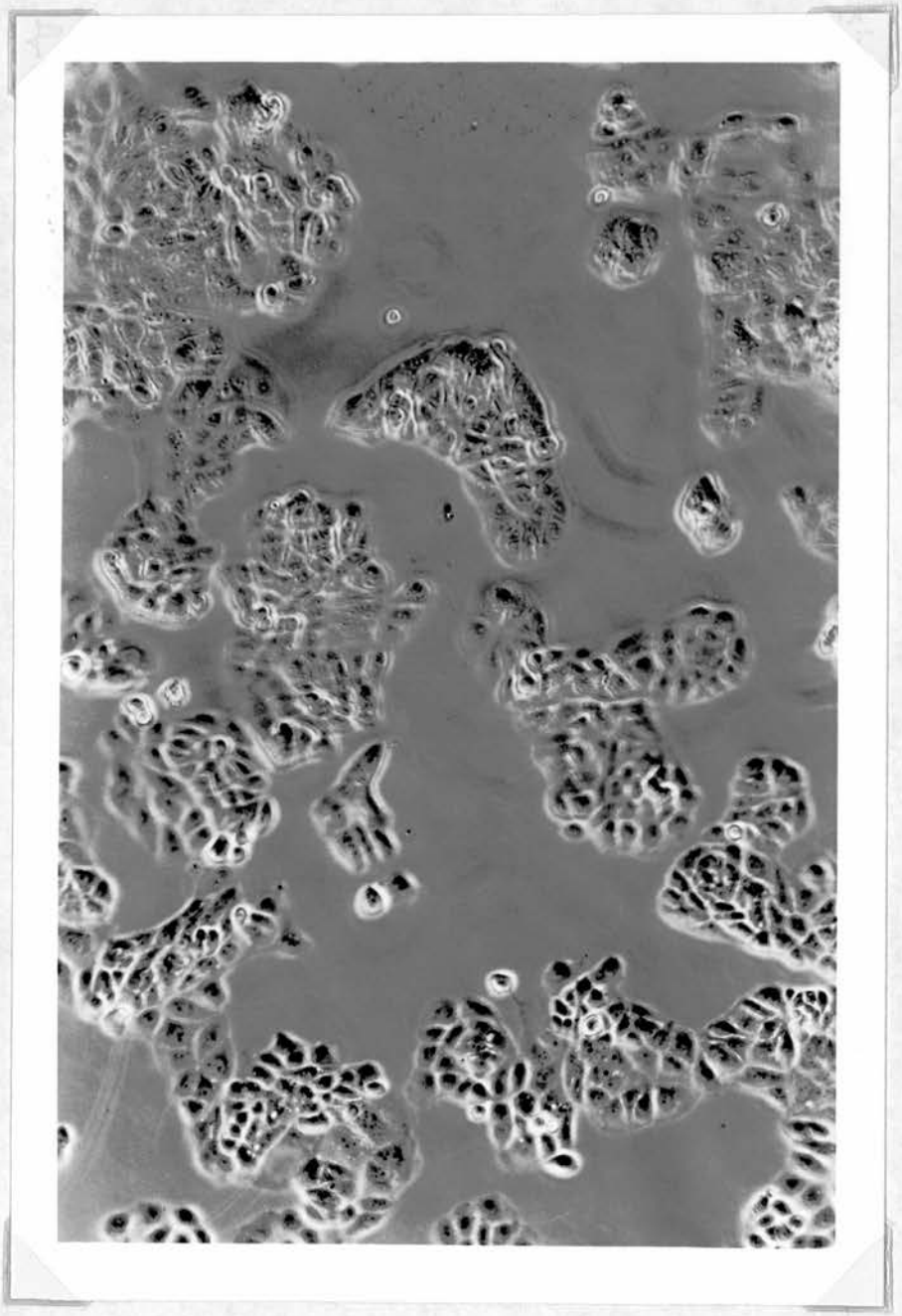


Figure 2.2

Photograph of the PEO4 ovarian carcinoma cell line in mid-log phase (x 125)

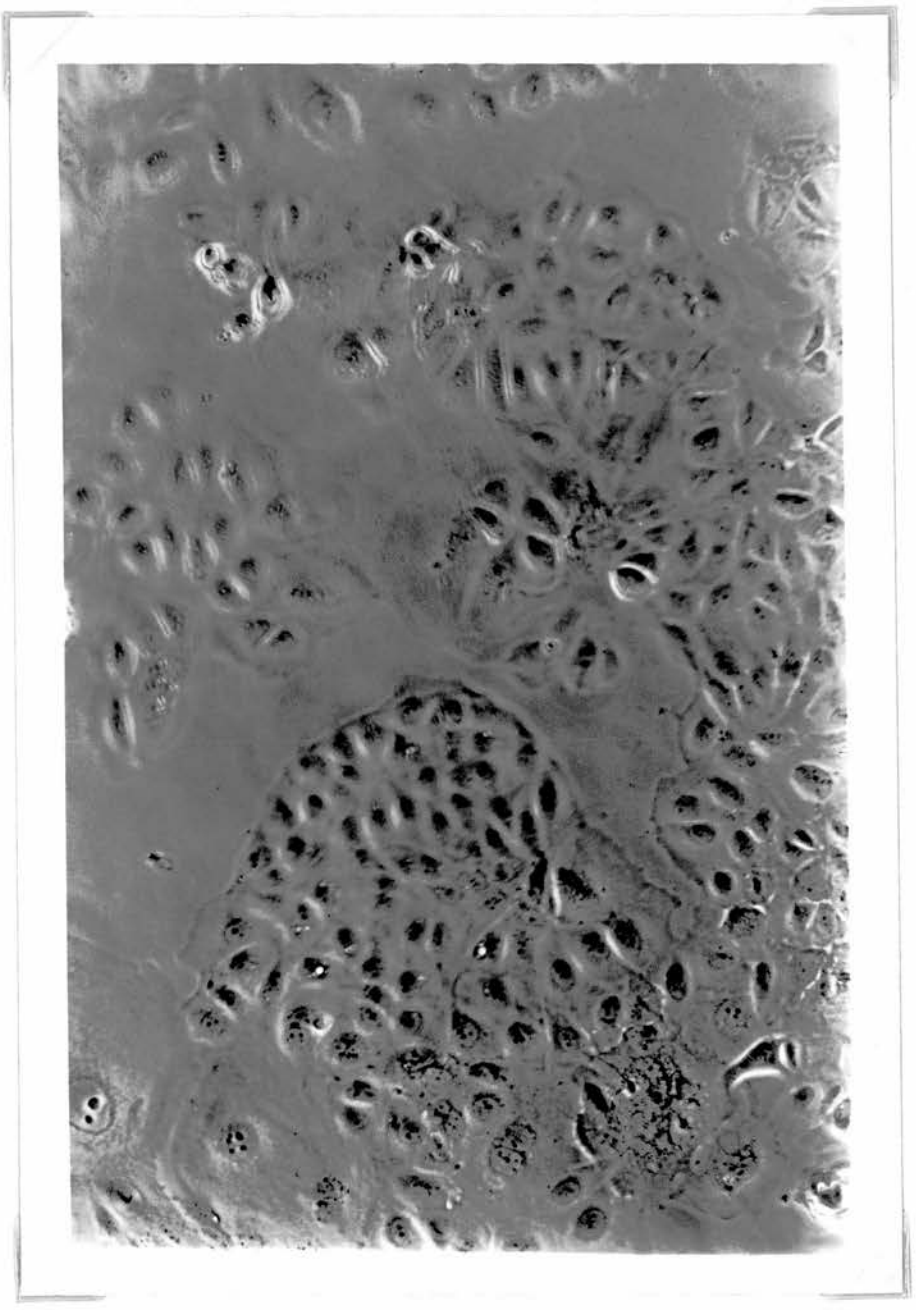


Figure 2.3

Photograph of the PEO14 ovarian carcinoma cell line in mid-log phase (x 125)

(ii) *In vivo* experiments

The two ovarian cancer xenograft models, PEO4 and HOX60, and two breast cancer xenografts, ZR-75-1 and T1068 discussed in this thesis were established in female nude (*nu/nu*) mice acquired from OLAC, Oxford, UK.

17 β-oestradiol slow-release pellets were obtained from Innovative Research of America.

2.1.2 Oestrogen receptor (ER) / Progesterone receptor (PR) measurements

Tris buffer	Sigma
ER and PR Enzyme Immunoassay (EIA) kits	Abbott Laboratories

2.1.3 Flow cytometry

FACScan flow cytometer	Becton Dickinson

(i) pS2 measurements

Anti-pS2 antibody	Histo-CIS
Sheep anti-mouse FITC conjugate	Sigma
Tween 20	Sigma

(ii) IGF-I receptor measurement

Anti-IGF-I Receptor antibody	Oncogene Science
Sheep anti-mouse FITC conjugate	Sigma

(iii) Cell cycle analysis

Propidium iodide	Fluka chemicals Ltd

2.1.4 Radioimmunoassay (RIA) of TGF-α and EGF

Anti-EGF (raised in sheep to purified human EGF)	Dr H Gregory, ICI (kindly supplied by Dr F.Habib Western General Hospital, Edinburgh)
Anti-sheep (donkey) IgG	Scottish Antibody Production Unit, Carluke
Bovine Serum Albumin	Sigma
EDTA	Sigma
Human recombinant EGF	Sigma
Human ^{125}I EGF, specific activity >28TBq/mmol	Amersham International PLC
Leupeptin	Sigma
Pepstatin A	Sigma
Phenyl methylsulphonyl fluoride (PMSF)	Sigma
TGF-α RIA kit	Peninsula Laboratories, Inc
Human recombinant TGF-α	Sigma

2.1.5 HSP27 Enzyme linked immunosorbent assay (ELISA)

D5 antibody	Kindly supplied by Prof. Roger King, Univ. of Surrey
Dithiothreitol	Sigma
Hydrogen peroxide (30%)	Sigma
HSP27 recombinant protein	Sigma
Microtitre Immulon 4 96-well plates	Dynatech
Peroxidase conjugated rabbit anti-mouse Immunoglobulin, polyclonal	Dako
Tetramethylbenzidine (TMB)	Sigma
Tris/HCl	Sigma
Tween 20	Sigma

2.1.6 Protein measurement

Bovine plasma albumin	Sigma
96 multi-well microtitre plates	Corning
Protein assay dye reagent	Bio Rad
Pyrex 12x75cm tubes	Corning

2.2 METHODS

2.2.1 Routine culture of cell lines *in vitro*

(i) Growth of cell lines.

Ovarian cancer cells were routinely maintained at 37°C in a humidified atmosphere of 5% CO_2 in Roswell Park Memorial Institute (RPMI) 1640 media containing phenol red indicator. This was supplemented with 10% heat-inactivated foetal calf serum (FCS; heat inactivated for 20 minutes at 56°C), streptomycin (100μg/ml) and penicillin (100 IU/ml).

ZR-75-1 and MDA-MB-231 breast cancer cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with phenol red indicator and supplemented with the above additives plus 2 mM L-glutamine.

(ii) Harvesting of cell lines

Cells at 70-80% confluence in 175cm^2 flasks were harvested by removing spent media and washing monolayers twice with pre-warmed PBS. Cells were then removed by addition of a 1:1 solution of trypsin (0.25% w/v in Gibco solution A) and versene (1mM EDTA in PBS, 0.5% v/v phenol red). This solution was deactivated after approximately 5 minutes by the addition of a small quantity of the appropriate media containing 10% FCS. Cells were passed through either a 19.5 (PEO14) or 21.5 gauge needle to produce a single cell suspension and then seeded into new flasks.

(iii) Freezing and storage of cells

Cells were frozen for later use in the following way: at 80-90 % confluence cells were trypsinised as described above and a single cell suspension produced. An aliquot was removed for counting by haemocytometer and the suspension centrifuged at 1000*g* for 10 minutes. The pellet was resuspended in ice cold 10% v/v DMSO/newborn calf serum at a concentration of $5x10^6$ cells/ml. Aliquots (1ml) were kept at -80°C overnight before final storage in liquid nitrogen.

Cells were recovered by thawing aliquots rapidly in warm water and the DMSO/cell suspension placed into either RPMI or DMEM containing 10% FCS, followed by centrifugation at 1000*g* for 10 minutes. Cell pellets were resuspended in the appropriate media and seeded into $25cm^2$ flasks which were incubated for 24 hours to allow attachment. Fresh medium was added after washing monolayers with PBS to remove dead cells. Cells for experiments were used at similar passage numbers.

(iv) Removal of oestrogens from culture media

For all *in vitro* experiments, cells were cultured in conditions free from oestrogens. The pH indicator in tissue culture medium, phenol red, has been previously shown to have weak oestrogenic effects (Berthois *et al.*, 1986), consequently cells were fed with phenol red-free media, either RPMI or DMEM which was supplemented with streptomycin (100μg/ml), penicillin (100 IU/ml), 2 mM L-glutamine and 5% double-charcoal stripped FCS.

Steroids were stripped from FCS as follows using a method adapted from Stanley *et al.*, (1977):

For 100ml of heat-inactivated FCS, a suspension of 1g charcoal/5mg dextran T70 in 10ml distilled H_2O (dH_2O) was prepared and stored at 4°C until use. The FCS was incubated with 250 units of sulphatase in a 37°C water bath for 2 hours. The pH of the serum was then adjusted to 4.2 with 2M HCl followed by addition of 5ml of the charcoal/dextran solution. This was stirred overnight at 4°C and then the dextran/charcoal removed by centrifugation at 18,000*g* for 20 minutes. The remaining 5ml of dextran/charcoal was added and the method repeated. The pH of the FCS was adjusted to 7.2 with 2M NaOH and sterilised by passing through 0.22μM filters. The double charcoal-stripped FCS was stored at -20°C until required.

2.2.2 Establishment of cell lines *in vivo*

Female nude (*nu/nu*) mice were maintained on a standard diet in negative pressure isolators (La Calhene, Cambridge, UK). Animals were at least 8 weeks old when experiments commenced. Xenografts were initiated from the PEO4 ovarian and ZR-75-1 breast cell lines by s.c. injection of cells into the flanks of female nude mice and once established used at passages 3-10 for experiments. The HOX60 xenograft originated from a patient with ovarian endometrioid adenocarcinoma and the T1068 xenograft was derived from a breast adenocarcinoma.

The ZR-75-1 xenograft was grown in the presence of a 1.7 mg 60 day-release 17 β-oestradiol pellet but the other xenograft models were maintained without supplement.

2.2.3 Growth studies

(i) Measurement of the effects of oestrogen on cell growth *in vitro*

The effects of 17 β-oestradiol on cell proliferation were determined in the nine ovarian and two breast cancer cell lines. Cells growing in mid-logarithmic phase were harvested by trypsinisation and a single cell suspension made in RPMI (ovarian lines) or DMEM (breast lines) media. Cells were plated using a 12ml Eppendorf multi-dispensor at densities of either 2.5 x 10^4 cells/well (PEO14) or 5 x 10^4 cells/well in 24-well plates. After 24 hours the media was removed, each well gently washed with warmed PBS and the media changed to phenol red-free RPMI or DMEM containing 5% double charcoal stripped FCS. In order to reduce effects of any remaining phenol red or steroids from FCS, plates were incubated for a further 24 hours. Subsequently (this time was designated day 0), the medium was replenished to that with or without 17 β-oestradiol at a range of concentrations from 10^{-12}M to 10^{-5}M. A stock solution of 17 β-oestradiol was prepared in absolute ethanol (10^{-2}M) from which successive dilutions were made up in the appropriate media. The trace amounts of ethanol in the experimental doses have no effect on the cell lines (Langdon *et al.*, 1990). The medium was renewed after 3 days with fresh media containing the required conditions. Quadruplicate wells were set up for each condition. On days 0, 3 and 6, cells were trypsinised and counted by Coulter counter.

a) Measurement of cell numbers

Counting of cell numbers was performed with a ZF Coulter Counter fitted with a 200μM probe.

Counting pots containing 9.8ml NaCl (0.9%) were prepared and bubbles allowed to disperse whilst cells were being trypsinised. Media was removed from plate wells and each one washed gently with PBS. A solution of trypsin/versene was added (250μl/well) and cells incubated for approximately 5 minutes at 37°C until detached. A single cell suspension was produced by passing cells through a 19.5 (PEO14) or 21.5 gauge needle (all other cells). An aliquot (200μl) was removed from each well and added to a counting pot containing 9.8ml of NaCl and the solution mixed. 500μl of this cell suspension was counted by the Coulter counter. Triplicate counts were recorded from each suspension. Cell number was calculated by multiplying the mean of triplicate counts by a dilution factor of 25 to give the number of cells per well. Final values for each experimental condition were determined from the mean number of cells in four wells.

(ii) Measurement of the effects of oestrogen on cell growth *in vivo*

To determine the effects of oestrogen on the growth of ovarian cancer cells *in vivo*, fragments of either the PEO4 or HOX60 xenografts were implanted subcutaneously into mice and allowed to reach a mean volume of $32mm^3$ after approximately one month. Animals (5-8) were allocated to either control or treatment groups and 30-day slow-release pellets of 17 β-oestradiol (1.7mg) implanted into the opposite flank to

that bearing the xenograft. Tumours were measured weekly using calipers and the volume calculated in mm^3 according to the formula;

$$\text{volume (V)} = \pi /6 \times l \times w^2$$

where l = the longest diameter and w = the diameter perpendicular to l.

2.2.4 Steroid receptor studies

(i) Effect of oestrogen on steroid receptors *in vitro*

Modulation of oestrogen receptors (ER) and progesterone receptors (PR) by 17 β-oestradiol was assessed in the nine ovarian and two breast cancer cell lines. Media from mid-log phase cells growing in $175cm^2$ culture flasks were removed and monolayers washed twice with pre-warmed PBS. Cells were fed with 50ml/flask of either phenol red-free RPMI or DMEM media. After 24 hours (day 0) media were replaced with phenol red-free media with or without 10^{-10}M 17 β-oestradiol. Cells were refed on day 3 and harvested on day 6 by removing the media and adding a small quantity of ice-cold PBS. Cells were then manually detached using a cell scraper. The cell/PBS suspension was removed from one flask to another identical flask and the procedure repeated until four flasks had been pooled. The cell suspension was centrifuged at 1000*g* for 10 minutes (4°C) to provide a single pellet for analysis by an Abbott enzyme immunoassay kit.

(ii) Effect of oestrogen on steroid receptors *in vivo*

Fragments of the PEO4 and HOX60 ovarian xenografts were implanted subcutaneously into the flanks of adult female nude (*nu/nu*) mice and allowed to

reach a mean volume of $32mm^3$ after approximately one month. Animals were randomly assigned to either control or treatment groups (5-8 mice each). Treatment consisted of subcutaneous implantation by trocar of a 1.7mg 17 β-oestradiol slow-release pellet into the flank of each mouse. After 60 days tumours were removed and oestrogen and progesterone receptor levels analysed as described below.

(iii) Enzyme immunoassay

ER and PR assays were kindly performed by Ann Tesdale and Tony Hawkins at the Department of Surgery, Royal Infirmary, Edinburgh. Xenograft tissue and cellular pellets were weighed and homogenised in buffer (10 mM Tris, 0.25 M sucrose, 1 mM EDTA, 22°C, pH 8.0) plus v/v 1% monothioglycerol and 10% glycerol. Following this, samples were centrifuged at 105,000*g* for 1 hour at 4°C. Cytosol protein concentrations were determined by the method of Bradford, (1976). Samples were analysed using Abbott ER and PR solid phase enzyme immunoassay kits according to the manufacturers instructions.

2.2.5 Analysis of pS2 expression by flow cytometry

(i) Preparation of cells

Cells growing in mid-logarithmic phase were extracted by trypsinisation as described previously. A single cell suspension was made and an aliquot counted using a haemocytometer. Cells were plated in six well plates by Eppendorf multi-dispensor at densities of 10^5 cells/well (PEO14) or 2 x 10^5 cells/well. After 24 hours media were removed and wells washed gently with PBS. Fresh, phenol red-free medium was

added and plates incubated for a further 24 hours. Cells were then treated with a single concentration of 10^{-10}M 17 β-oestradiol (day 0). Cells were either trypsinised and analysed on day 3 or refed and analysed on day 6.

(ii) Analysis of pS2 by flow cytometry

After trypsinisation cells were fixed in 70% ethanol, 4°C for 30 minutes, and then washed twice in PBS buffer containing 5% FCS/0.5% Tween 20. Anti-pS2 antibody (100µl) diluted 1:2 in buffer was added to cells on ice and left for 30 minutes. After washing in buffer, cells were exposed to 100µl of a FITC-conjugated sheep anti-mouse antibody (diluted 1:20 in buffer) for 30 minutes. Cells were then washed once in buffer, twice in PBS before being resuspended in PBS ready for reading by flow cytometer (*see below). Samples were analysed using a FACScan instrument (Becton Dickinson) equipped with an argon laser emitting an excitation wavelength of 488nM. 10,000 events were collected for each sample and the data evaluated using Lysys II software (Becton Dickinson).

2.2.6 Cell cycle analysis

* Cell cycle analysis was performed in conjunction with the pS2 experiments. Directly before cells were analysed on the FACScan, 100µl of propidium iodide (100µg/ml) was added and samples incubated at room temperature in the dark for 15 minutes. Cells were then analysed by flow cytometer.

2.2.7 Analysis of IGF-I receptor modulation by oestrogen

The modulation of the IGF-I receptor by oestrogen was determined by flow cytometry. Cells were prepared as described for analysis of pS2 expression. After six days exposure to 10^{-10}M 17 β-oestradiol in six well plates, cells were detached by trypsinisation, resuspended and fixed in 70% ethanol at 4°C for 30 minutes. Cells were washed in cold PBS and incubated with 100μl of anti-IGF-I receptor antibody (diluted 1:10 in PBS/5%FCS/0.5% Tween 20 wash buffer) for 30 minutes on ice. Samples were then washed once with the wash buffer and incubated with 100μl of a sheep anti-mouse FITC-conjugate antibody for 30 minutes on ice. Cells were washed twice in cold PBS and resuspended in 1ml of PBS for analysis using the FACScan flow cytometer.

2.2.8 Analysis of HSP27 expression

(i) Preparation of pellets from routinely cultured ovarian and breast cancer cell lines

To determine the HSP27 expression levels in routinely cultured cell lines, pellets were prepared from cells growing at 70-80% confluency in 25cm^2 flasks. Monolayers were washed twice with PBS and cells manually removed in a small quantity of PBS using a scraper. Samples were spun at 1000*g* for 10 minutes at 4°C and the PBS removed. Cell pellets were frozen at -80°C until required.

(ii) Preparation of xenograft samples

PEO4, HOX60, ZR-75-1 and T1068 xenografts were established as described previously in female nude mice. Tumours were removed when approximately 1.5-2.0cm diameter and frozen in liquid nitrogen until later use.

Cell pellets and xenograft samples were initially analysed for HSP27 expression by Professor Roger King at the University of Surrey. Measurements of constitutive HSP27 expression in the cell lines was confirmed when investigating the modulation by oestrogen.

(iii) Modulation of HSP27 by 17 β-oestradiol *in vitro*

To investigate the effects of 17 β-oestradiol on HSP27 expression, cell lines growing in mid-log phase in 175cm^2 flasks were washed twice with pre-warmed PBS, and media changed to phenol red-free media containing 5% dcsFCS. Cells were refed 24 hours later and exposed to a 10^{-10}M concentration of 17 β-oestradiol. After 72 hours cells were harvested as described previously by scraping, and then pelleted and stored at -80°C until required.

(iv) Modulation of HSP27 by 17 β-oestradiol *in vivo*

Fragments of xenografts were subcutaneously implanted into the flanks of female nude mice in a similar manner as that described for ER and PR analysis. When tumours reached an approximate size of 32mm^3 in volume, animals were allocated randomly to control (receiving no treatment) or treated groups (17 β-oestradiol was administered via a 60 day slow-release pellet). At the end of this period tumours from control and treated animals were extracted and stored in liquid nitrogen until use.

(v) Cytosol preparation

HSP27 was determined in cytosols of cells pellets or xenografts prepared in the following way:

Xenografts were weighed and homogenised with 5 volumes of Tris/EDTA buffer (10mM Tris/HCl, 1mM EDTA, 2mM dithiothreitol, pH 7.4) on ice for 2 x 20 seconds using a Silverson homogeniser, allowing cooling between bursts. Pellets from cell lines were resuspended in 0.5ml of Tris/EDTA buffer and homogenised on ice by hand using a glass homogeniser. Samples were then spun at 200,000*g* at 4°C for 1 hour, cytosols removed for analysis and the pellet discarded.

(vi) Protein concentration estimation

Cytosol protein levels were determined by a colourimetric assay (BioRad) adapted from Bradford, (1976). Duplicate tubes were set up containing 20µl of either the unknown sample or BSA protein standards ranging from 0.18-1.4 mg/ml. Two BSA protein quality controls were also set up at 1:100 and 1:200 dilutions (0.8 and 0.4 mg/ml). BioRad Protein Dye assay reagent was diluted in distilled H_2O (1:5), mixed and filtered through Whatman No. 1 paper. The dye solution was then added to the samples, 1ml/tube, and the solutions vortexed. Aliquots (200µl) from each tube were loaded into a 96 flat-bottomed-well microtitre plate and read at 600nm using a BioRad EIA plate reader which had been calibrated to zero. Protein concentrations were determined by comparison with the standard curve readings. The mean of two separate readings for each sample was calculated for use in the ELISA.

(vii) Analysis by ELISA

HSP27 expression was detected in sample cytosols by an ELISA method (Cano *et al.*, 1986) using a monoclonal antibody D5, kindly provided by R. King, and incorporating cytosolic myometrium (30, 20, 10, 5 μg/ml) and recombinant HSP27 (50, 25, 10 μg/ml) diluted in PBS as standards. Essentially, duplicate samples diluted in PBS to give 40, 20 and 10μg/ml (ovarian cell cytosols) or 6.25, 5.00, 3.75 and 2.5 μg/ml (breast cell cytosols) were plated in Immulon 4 microtiter plates at 200μl per well and incubated at 37°C for 90 minutes. Plates were washed 3 times in PBS, 0.05% Tween buffer and then treated with either the D5 antibody (0.75μg/ml, 200μl/well) or 200μl PBS/Tween buffer (control wells) for 2 hours at 37°C. After washing 3 times in buffer, wells were incubated with 200μl of peroxidase-conjugated rabbit anti-mouse immunoglobulin at 1:1000 at 37°C for 1 hour. Following further washing, 200μl of the peroxidase substrate, tetramethylbenzidine, was added to each well for 4 to 10 minutes in the dark. The peroxidase substrate was aliquoted from a stock solution containing 100μl of tetramethylbenzidine (20 mg/ml in DMSO) per 20ml of substrate buffer (0.05M Na_2HPO_4 (anhydrous) / 0.02M citric acid, pH 5.0), plus the addition of 14μl of 30% hydrogen peroxide when ready to use. Reactions were terminated with 50μl/well of 0.5M sulphuric acid and plates read at 450nm using a BioRad plate reader. Readings were calibrated by initially zeroing the reader with a blank micro-titre plate.

HSP27 expression was calculated in the unknown samples per μg/protein by quantifying against the value obtained for 1μg of either a myometrial or recombinant HSP27 protein standard run at the same time.

2.2.9 Measurement of EGF and TGF-α production by radioimmunoassay

(i) Preparation of conditioned media

The effects of 17 β-oestradiol on the production of TGF-α and EGF by the cell lines was determined using TGF-α RIA kits (Peninsula Labs Inc.) and an EGF radioimmunoassay method adapted from McDonald *et al.*, (1990). Conditioned medium was prepared for analysis as follows: Cells growing in mid-logarithmic phase in 175cm^2 culture flasks were washed twice with pre-warmed PBS and 50ml per flask of phenol red-free/serum-free media added. Flasks were incubated for 24 hours followed by exposure to 50ml of fresh media containing 17 β-oestradiol (10^{-10}M). Cells were then incubated for 72 hours. After this time, media from the flasks were removed and pooled to provide 100ml per condition. This was spun at 4°C, 1000*g* for 10 minutes to remove any cells and subsequently transferred to round-bottomed flasks on ice. Protease inhibitors were added to give final concentrations of 0.2mM phenylmethanesulphonyl fluoride, 1mM EDTA, 0.5 mg/L leupeptin and 0.7 mg/L pepstatin A (adapted from Stromberg *et al.*, 1992). Media were concentrated by prior immersion in a methanol/CO_2 bath followed by freeze-drying using an Edwards freeze drier. Once fully dry, samples were resuspended in dH_2O to produce a 50x concentrate. This was then dialysed in benzoylated cellulose dialysis membrane tubing against PBS (2 L) for 24 hours at 4°C to remove excess salts with a molecular weight

of <1200. Concentrates were freeze-dried again and resuspended in an appropriate volume of dH_2O to produce a final concentration 100 times greater than that of the original media before processing. Samples were dialysed again at 4°C for 48 hours, renewing the PBS after 24 hours, and stored at -80°C before analysis.

(ii) Radioimmunoassay

a) Transforming Growth Factor-alpha (TGF-α)

Analysis of TGF-α was performed using a competitive radioimmunoassay kit incorporating a rabbit anti-TGF-α (rat) antibody and rat TGF-α standard. A standard curve was constructed using a concentration range of peptide from 1.0 to 128pg/tube (100µl). All samples were diluted in the RIA kit buffer. Unknown samples at 100x and 50x concentration (1:2 dilution of 100x concentrate) were aliquoted (100µl/tube) and 100µl of rabbit anti-TGF-α antibody added to both the standards and unknowns. Tubes were vortexed and left at 4°C overnight.

The following day ^{125}I-TGF-α stock was prepared in buffer to give a count of 10,000-15,000 cpm/sample. Tracer (100µl) was then added to each sample, tubes vortexed and left for a further 24 hours at 4°C. After this period, 100µl of goat anti-rabbit IgG serum was added to each tube, together with 100µl of normal rabbit serum. Tubes were vortexed and incubated at room temperature for 90 minutes. RIA buffer was then added to each tube (500µl), vortexed, and the samples centrifuged at 1700*g*, 4°C for 20 minutes to separate bound and free ^{125}I-TGF-α. Tubes were carefully aspirated by pasteur pipette and the pellet containing the bound ^{125}I-TGF-α counted for 1 minute on a Cobra AutoGamma counter (Packard Canberra).

Samples were determined in duplicate at each concentration and non-specific binding (NSB) estimated in the absence of primary antibody and unlabelled TGF-α. Total binding was determined by incubation of the tracer in the absence of cold TGF-α (ie. sample or standard). Maximum binding (Bo) was calculated from this value minus the NSB value. TGF-α activity in the samples was determined from a standard curve, constructed by plotting %Bound/Bo against the log of the standard concentrations.

b) Epidermal Growth Factor (EGF)

Analysis of EGF in conditioned media was determined by a liquid phase competitive radioimmunoassay adapted from McDonald *et al.*, (1990). All solutions were made up in 0.01M PBS. A standard curve was prepared using EGF concentrations ranging from 0.2-200 ng/ml. Samples were used at x100 and x50 concentrations and human urine incorporated as a quality control.

Standards or samples (100µl/tube) were aliquoted and to each tube was added 100µl of sheep anti-human EGF polyclonal IgG diluted to 1:30,000 and 100µl of ^{125}I EGF (15,000cpm). Tubes were vortexed and incubated at 37°C for 2 hours. Donkey anti-sheep polyclonal IgG (diluted 1:20 in 1:200 normal sheep serum) (250µl) was then added and the samples incubated overnight at 4°C. Following this, 500µl aliquots of dH_2O were added to each tube, the samples vortexed and centrifuged at 3500 rpm to separate bound and free ^{125}I EGF. Tubes were then carefully aspirated and the pellet containing the bound ^{125}I EGF counted for 1 minute in a Cobra AutoGamma Counter.

EGF activity was determined from a standard curve plot in a similar way to that described for TGF-α analysis.

3. RESULTS

3.1 Modulation of growth by oestrogen

The following section describes the results from experiments designed to assess the effects of oestrogen on the *in vitro* growth of nine ovarian carcinoma cell lines and to determine any correlation between the oestrogen sensitivity of these lines and their ER status. In addition, the effects of oestrogen on the growth of two ovarian carcinoma xenografts, PEO4 and HOX60 are presented.

The nine ovarian carcinoma cell lines possess a variety of oestrogen receptors (ER) levels (Table 2.1) (Langdon *et al.*, 1990). Three of the lines, PEO14, PEO16 and PEO23 express receptor levels of less than 5 fmol/mg protein, the value considered to be at the limit of detection. These lines are therefore regarded as ER-negative. The remaining lines are ER-positive, ranging from relatively low levels of 12 to 23 fmol/mg protein for the cell lines PEA1 and PEA2, to moderate-high ER levels of 90 to 130 fmol/mg protein for the PEO1, PEO4, PEO6, cell lines.

3.1.1 Effects of oestrogen on proliferation *in vitro*

Growth response curves were initially produced for the PEO4 and PEO14 ovarian cell lines, and two breast cell lines, ZR-75-1 and MDA-MB-231 using two concentrations of 17 β-oestradiol (10^{-10}M and 10^{-8}M), to confirm the ability of oestrogen to modulate the growth of ER-positive cell lines but not ER-negative lines. Secondly, the oestrogen sensitivity of eight of the ovarian cell lines was investigated by exposing cells to a range of 17 β-oestradiol concentrations. Effects on cells were examined in the absence of phenol red indicator and in the presence of 5% dcsFCS.

(i) Effects of oestrogen on cell growth rate

The effects of oestrogen on the *in vitro* growth of breast carcinoma cell lines has been well recorded. Previous studies have shown the ER-positive ZR-75-1 cell line to be growth stimulated by oestrogen, whilst the ER-negative MDA-MB-231 cell line is not (Darbre and Daly, 1989, Lippman *et al.*, 1976). These two lines were included in this study to compare oestrogen effects between the two model systems, and to act as positive and negative controls for oestrogen sensitivity together with the ER-positive PEO4 and ER-negative PEO14 ovarian cell lines whose growth responsiveness to oestrogen has also been shown to correlate with their ER status (Langdon *et al.*, 1990).

To determine whether the oestrogen growth responses of the PEO4 and PEO14 ovarian cell lines were typical of those described previously, cells were exposed to 10^{-10}M and 10^{-8}M concentrations of 17 β-oestradiol for six days. The breast cancer cell lines, ZR-75-1 and MDA-MB-231 were similarly treated to determine their oestrogen responsiveness. Growth was determined by changes in cell number.

a) ZR-75-1

Figure 3.1 illustrates the effects of 17 β-oestradiol on the growth of the ER-positive breast cancer line, ZR-75-1. Oestrogen-deprived ZR-75-1 control cells showed little growth in these conditions as demonstrated by the lack of any significant change in cell number over the six day period. However, substantial growth modulations were observed with both 10^{-10}M and 10^{-8}M concentrations of 17 β-oestradiol after three and six days, producing a significant increase ($p<0.001$) in cell number as compared

to cells grown in the absence of oestrogen over the period of study. This is in agreement with previous data showing that this is an oestrogen sensitive cell line.

b) MDA-MB-231

The effects of 17 β-oestradiol on the growth of the ER-negative MDA-MB-231 breast carcinoma cell line after three and six days are shown in figure 3.2. There was a progressive increase in the number of MDA-MB-231 control cells cultured throughout the period of study. Treatment with either 10^{-10}M or 10^{-8}M concentrations of 17 β-oestradiol had no significant effect on cell growth as compared to control cells after either three or six days exposure. This result is consistent with the lack of expression of ER and is comparable with the growth response curve for the ER-negative PEO14 ovarian carcinoma cell line (see figure 3.4).

c) PEO4

Figure 3.3 shows a growth response curve, typical of three experiments, for the ER-positive PEO4 ovarian cell line after exposure to the two concentrations (10^{-10}M and 10^{-8}M) of 17 β-oestradiol. In contrast to ER-positive ZR-75-1 breast cancer cells, control PEO4 cells (cultured without oestrogen supplement) progressively increased in number over the six day period, showing a 30% increase after three days and rising to 70% after six days. Both concentrations of 17 β-oestradiol stimulated cell growth above that noted with control cells. A significant increase ($p<0.001$) in PEO4 cell number of approximately 70% was observed after three days exposure, rising to a 176 - 215 % increase after six days treatment; the 10^{-10}M concentration causing a slightly greater response. This result is comparable to previous reports of the

oestrogen sensitivity in the PEO4 cell line (Langdon *et al.*, 1990), where an increase in growth was noted after 4, 7 and 10 days exposure to both 10^{-10}M and 10^{-8}M concentrations of 17 β-oestradiol. Dose response curves (see figure 3.6) indicated that concentrations of oestrogen at 10^{-10}M, 10^{-9}M and 10^{-8}M were equipotent in producing a growth stimulation in PEO4, and thus the small differences between dose effects seen with the growth curves may be due to individual experiment variability. This is confirmed by repeat experiments of the PEO4 growth curve which show no significant differences between the growth effects produced by 10^{-10}M and 10^{-8}M 17 β-oestradiol.

d) PEO14

Figure 3.4 shows a growth response curve for the ER-negative PEO14 ovarian cell line after exposure to 10^{-10}M and 10^{-8}M 17 β-oestradiol over six days. Control PEO14 cells cultured in the absence of oestrogen increased in number after three days culture but less of an increase was seen during the rest of the time course. In contrast to ER-positive PEO4 cells, treatment of PEO14 cells with either concentration of oestrogen produced no stimulation of growth above that seen in control conditions after three or six days. This is in agreement with previous data, and is consistent with the negative ER status of the PEO14 cell line.

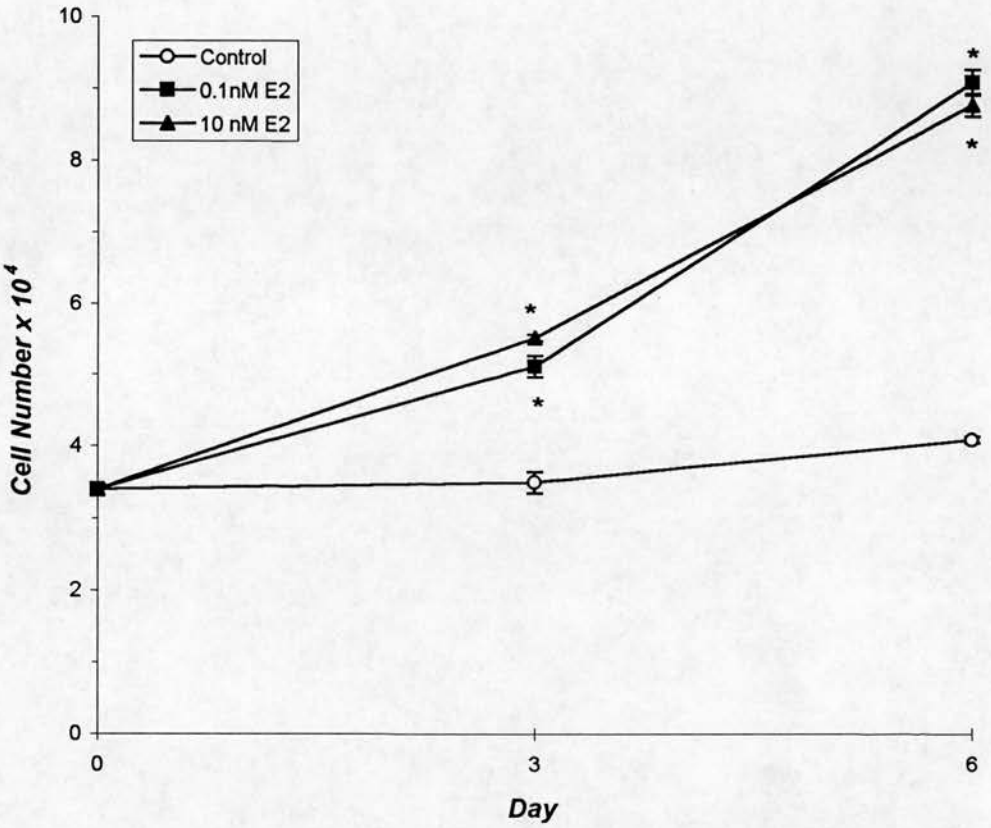


Figure 3.1

Effects of exogenous addition of 17 β-oestradiol (E_2) on the proliferation of the ZR-75-1 cell line over a period of six days. Cells were cultured in phenol red-free media supplemented with 5% dcsFCS and exposed to one of the indicated treatments on day 0. Media was replenished after 72 hours. Cells were removed by trypsinisation and counted by Coulter Counter after 0, 72 and 144 hours. Symbols represent the mean of quadruplicate samples from one experiment which is typical of at least two other experiments and bars represent standard error. *$p<0.001$ for the difference between control cells and the group indicated, as evaluated by a Student's *t*-test.

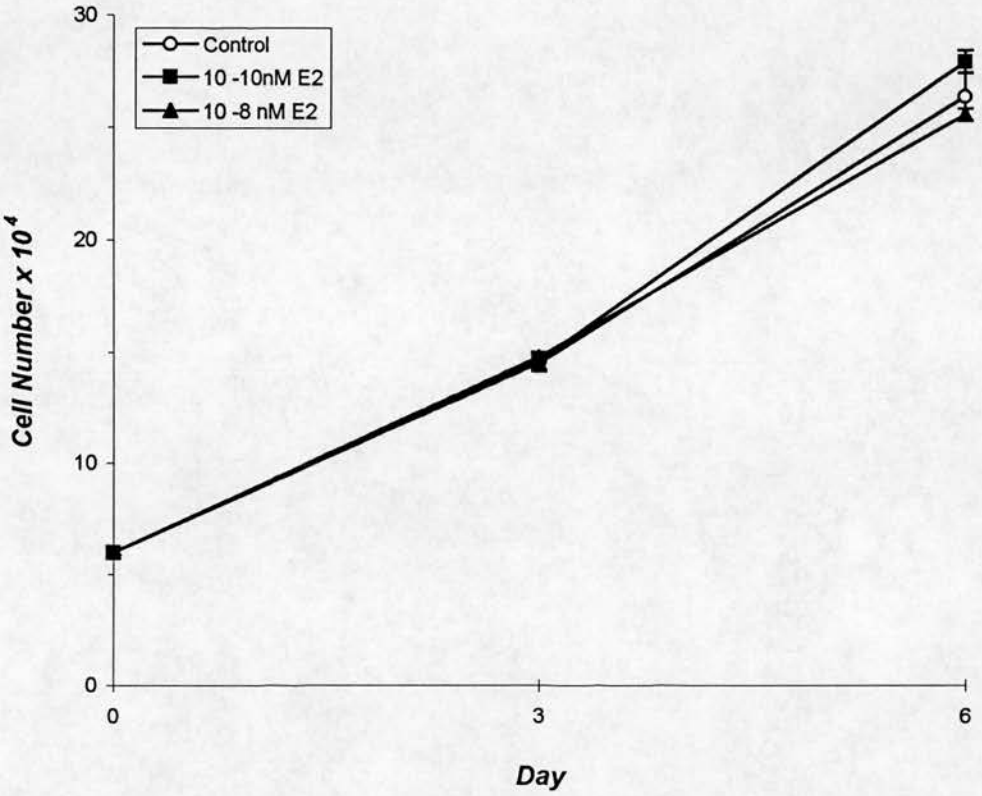


Figure 3.2

Effects of exogenous addition of 17 β-oestradiol (E_2) on the proliferation of the MDA-MB-231 cell line over a period of six days. Cells were cultured in phenol red-free media supplemented with 5% dcsFCS and exposed to one of the indicated treatments on day 0. Media was replenished after 72 hours. Cells were removed by trypsinisation and counted by Coulter Counter after 0, 72 and 144 hours. Symbols represent the mean of quadruplicate samples from one experiment which is typical of at least two other experiments and bars represent standard error.

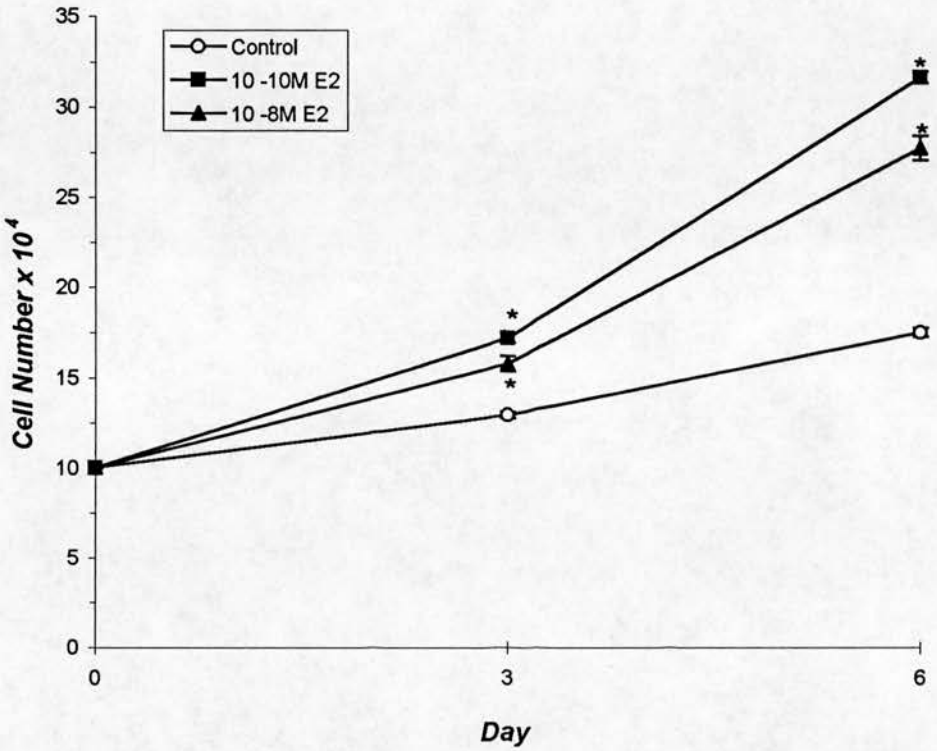


Figure 3.3

Effects of exogenous addition of 17 β-oestradiol (E_2) on the proliferation of the PEO4 cell line over a period of six days. Cells were cultured in phenol red-free media supplemented with 5% dcsFCS and exposed to one of the indicated treatments on day 0. Media was replenished after 72 hours. Cells were removed by trypsinisation and counted by Coulter Counter after 0, 72 and 144 hours. Symbols represent the mean of quadruplicate samples from one experiment which is typical of at least two other experiments, and bars represent standard error. *$p<0.001$ for the difference between control cells and the group indicated, as evaluated by a Student's *t*-test.

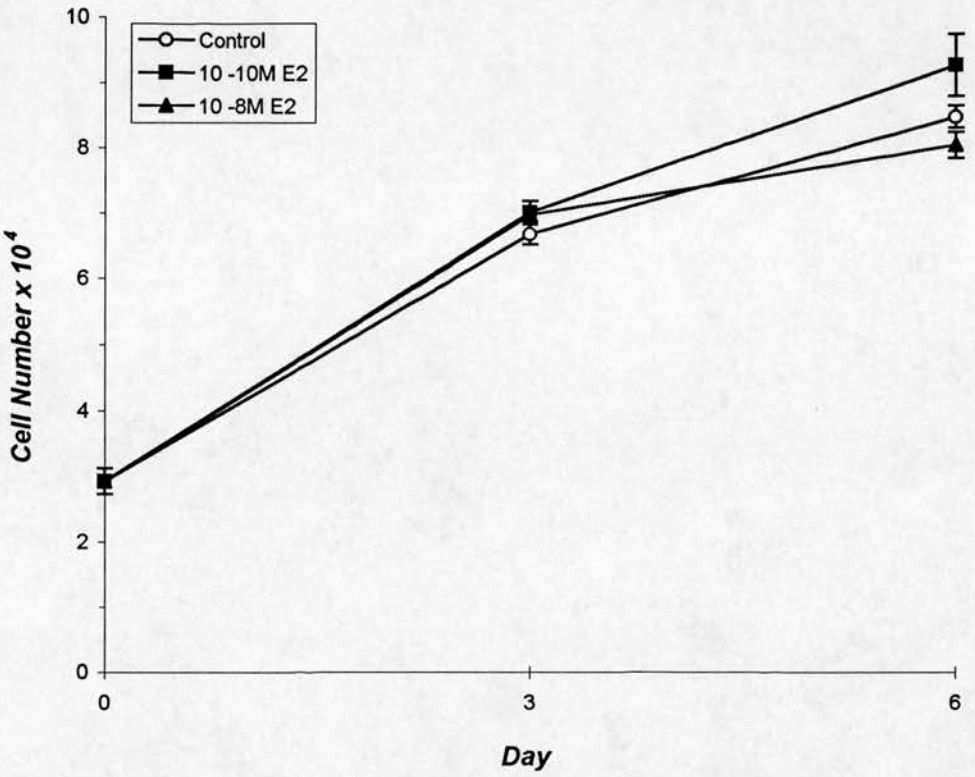


Figure 3.4

Effects of exogenous addition of 17 β-oestradiol (E_2) on the proliferation of the PEO14 cell line over a period of six days. Cells were cultured in phenol red-free media supplemented with 5% dcsFCS and exposed to one of the indicated treatments on day 0. Media was replenished after 72 hours. Cells were removed by trypsinisation and counted by Coulter Counter after 0, 72 and 144 hours. Symbols represent the mean of quadruplicate samples from one experiment which is typical of at least two other experiments, and bars represent standard error.

(ii) Effects of varying concentrations of 17 β-oestradiol on the growth of ovarian carcinoma cell lines

In order to obtain a more detailed profile of oestrogen sensitivity in ER-positive and negative ovarian cell lines, eight ovarian cell lines were exposed to a range of 17 β-oestradiol concentrations (10^{-12}M - 10^{-5}M) over a six day period. Cells were cultured in phenol red-free media supplemented with 5% stripped foetal calf serum plus the addition of one of the above concentrations of 17 β-oestradiol, trypsinised after six days and cell number determined by Coulter Counter.

a) PEO1 cell line

The effects of oestrogen on cell number for the ER-positive PEO1 cell line are illustrated in figure 3.5. All concentrations of 17 β-oestradiol (10^{-12} - 10^{-5}M) produced a significant increase ($p<0.001$) in the number of cells as compared to control values. Concentrations from 10^{-12} to 10^{-10}M produced a progressive increase in cell number reaching maximum stimulation at 10^{-10}M, approximately 140% higher than control cells. Increasing concentrations from 10^{-9} to 10^{-5}M produced a gradual decline in stimulation, the 10^{-5}M concentration producing the smallest increase in cell number above the control.

b) PEO4 cell line

Figure 3.6 shows the effect of 17 β-oestradiol on ER-positive PEO4 cells. Concentrations between 10^{-12}M and 10^{-6}M increased cell numbers significantly above control levels ($p<0.001$). A gradual increase was observed with concentrations from 10^{-12} to 10^{-9}M, with maximal stimulation of approximately 90% above control values

at 10^{-10}M and 10^{-9}M concentrations. This result parallels the magnitude of stimulation seen in the growth response curve for PEO4 cells treated with 10^{-10}M and 10^{-8}M 17 β-oestradiol after six days, shown in figure 3.3.

A progressive decrease in growth stimulation was observed at concentrations higher than 10^{-9}M, with a concentration of 10^{-5}M producing a significant decrease in cell numbers to approximately 40% of control values.

c) PEO6 cell line

The effects of 17 β-oestradiol on the ER-positive PEO6 cell line are shown in figure 3.7. Concentrations between 10^{-12}M and 10^{-7}M produced a significant increase in cell numbers which reached a maximum of approximately 40% above control cells with a concentration of 10^{-10}M 17 β-oestradiol. Similar to the PEO4 cell line, growth increases were less marked at higher concentrations (10^{-8} to 10^{-6}M), and a significant growth inhibition, 15% of the control value, was demonstrated with a concentration of 10^{-5}M.

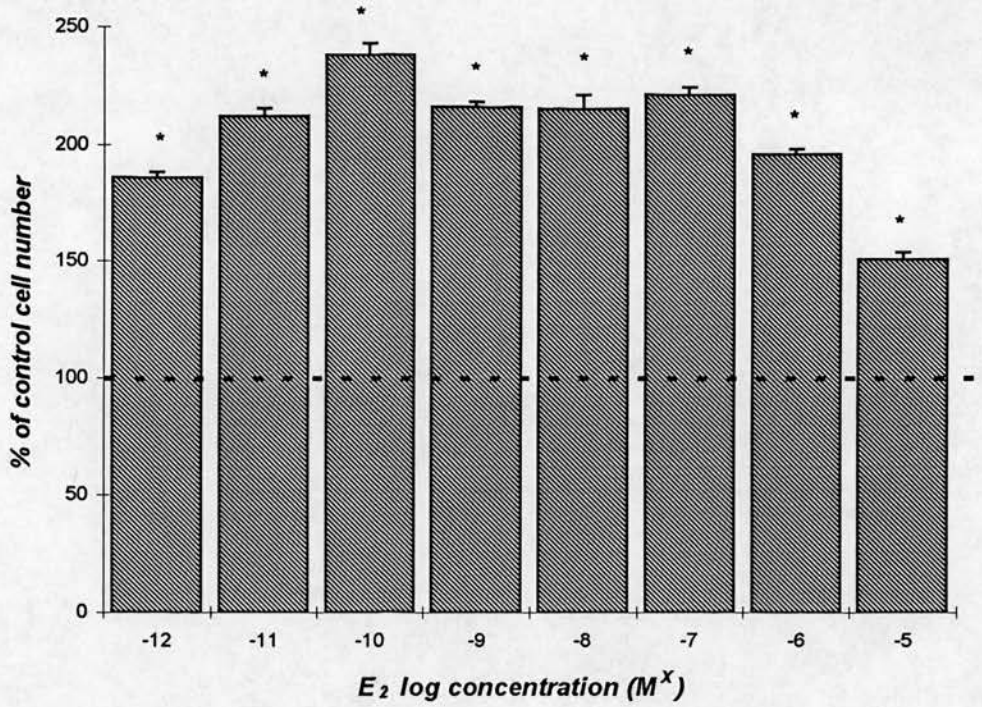


Figure 3.5

Effects of exogenous addition of 17 β-oestradiol (E_2) on PEO1 cell number. Cells were grown in phenol red-free media supplemented with 5% dcsFCS and treated with the concentrations of E_2 indicated over a period of six days. The graph shows the result from one experiment which is typical of at least three identical experiments. Each value represents the mean of quadruplicate samples, each sample being counted in triplicate by Coulter Counter. Error bars denote standard error. *$p<0.001$ for the difference between the control and the group indicated, as evaluated by a Student's *t*-test.

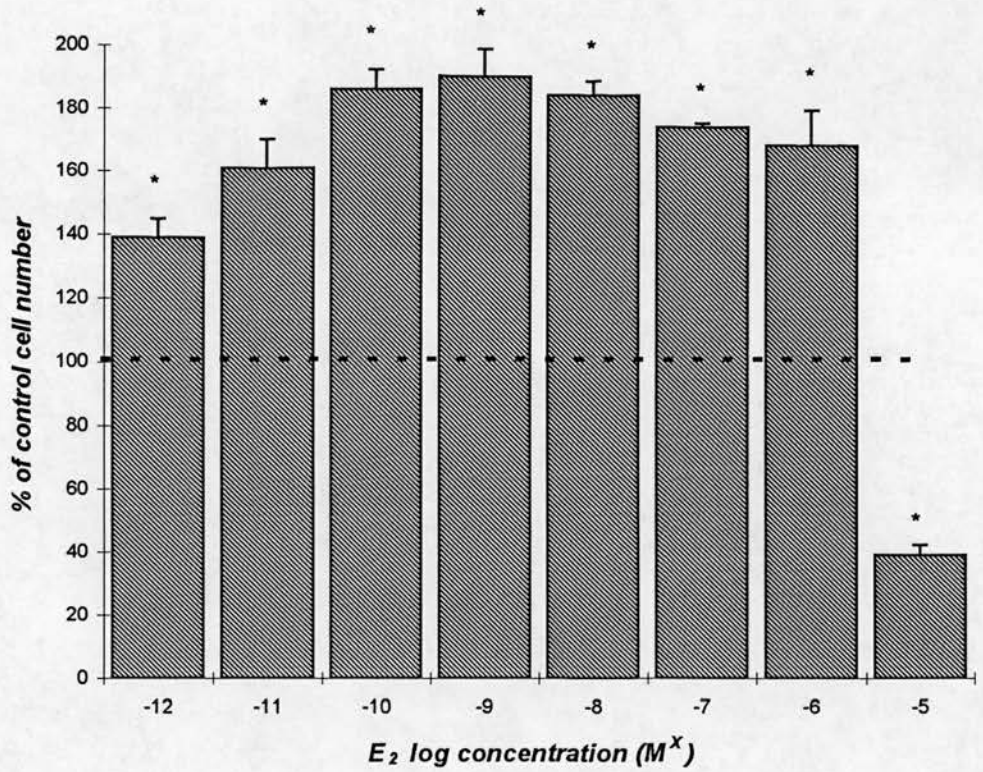


Figure 3.6

Effects of exogenous addition of 17 β-oestradiol (E_2) on PEO4 cell number. Cells were grown in phenol red-free media supplemented with 5% dcsFCS and treated with the concentrations of E_2 indicated over a period of six days. The graph shows the result from one experiment which is typical of at least three identical experiments. Each value represents the mean of quadruplicate samples, each of which was counted in triplicate by Coulter Counter. Error bars denote standard error. *$p < 0.001$ for the difference between the control and the group indicated, as evaluated by a Student's *t*-test.

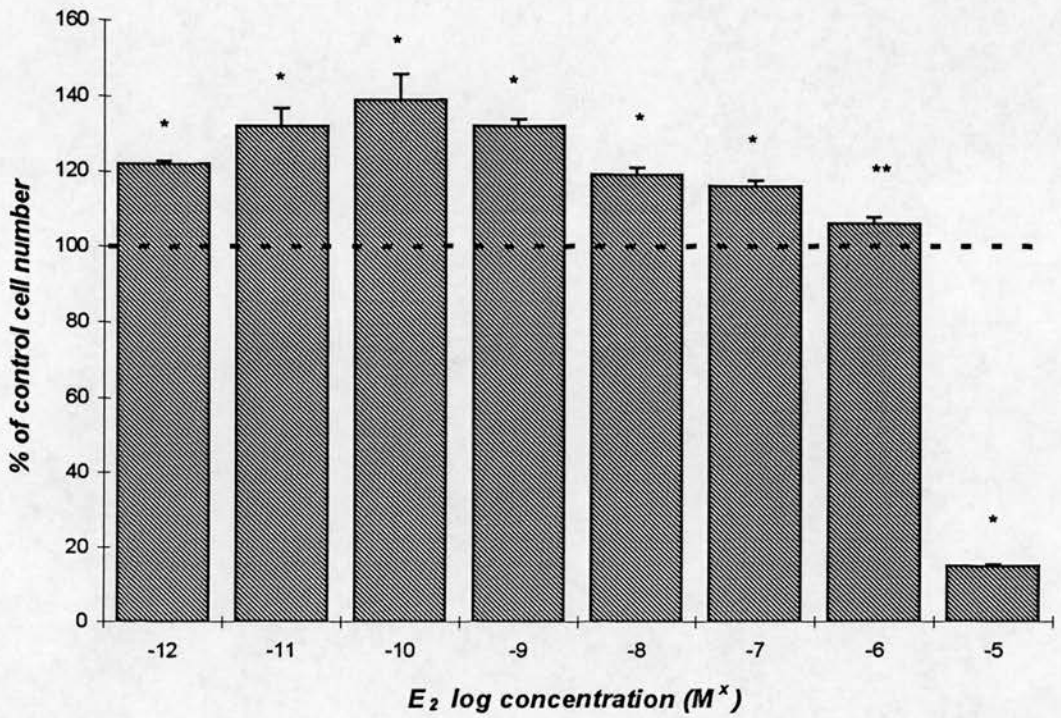


Figure 3.7

Effects of exogenous addition of 17 β-oestradiol (E_2) on PEO6 cell number. Cells were grown in phenol red-free media supplemented with 5% dcsFCS and treated with the concentrations of E_2 indicated over a period of six days. The graph shows the result from one experiment which is typical of at least three identical experiments. Each value represents the mean of quadruplicate samples, each of which was counted in triplicate by Coulter Counter. Error bars denote standard error. *$p<0.001$, **$p<0.05$ for the difference between the control and the group indicated, as evaluated by a Student's *t*-test.

d) PEA1 cell line

Figure 3.8 shows the effects of 17 β-oestradiol on the PEA1 cell line which expresses only a low level of ER. At concentrations between 10^{-12}M and 10^{-6}M there was no significant difference ($p>0.05$) between the number of treated and control PEA1 cells. This differs from the responses noted in the moderate-high ER status PEO1, PEO4 and PEO6 cell lines which are growth stimulated by these concentrations. At the 10^{-6}M and 10^{-5}M concentrations of 17 β-oestradiol there appeared to be a decrease in cell number, but this only achieved significance at the higher concentration, cell number reaching only 30% of the control value.

e) PEA2 cell line

The effects of 17 β-oestradiol on the PEA2 cell line which expresses low levels of ER is shown in figure 3.9. Concentrations of 17 β-oestradiol between 10^{-12}M and 10^{-6}M caused no significant change in cell number. However, at the highest concentration, 10^{-5}M there was a significant decrease in cell numbers to approximately 60% below control values. These observations are comparable to the result obtained with the PEA1 cell line.

The lack of response of PEA1 and PEA2 to levels of oestrogen which are stimulatory to both breast and ovarian carcinoma cell lines expressing moderate-high levels of ER, (similar to that observed with ER-negative lines below), leads to the conclusion that a certain level of ER expression is necessary in order to see growth stimulation by oestrogen.

f) PEO16 cell line

The effects of 17 β-oestradiol on the ER-negative PEO16 cell line are shown in figure 3.10. There was no significant effects ($p>0.05$) on cell number at concentrations between 10^{-12}M and 10^{-7}M. However, the two highest concentrations of 17 β-oestradiol (10^{-6} and 10^{-5}M) produced significant decreases in cell numbers. This result is consistent with the lack of oestrogen sensitivity of ER-negative lines noted previously with both the ER-negative PEO14 ovarian line and the MDA-MB-231 breast carcinoma cell line (Figures 3.4, 3.2).

g) PEO23 cell line

Concentrations of 17 β-oestradiol between 10^{-12} and 10^{-6}M produced no significant effects ($p>0.05$) on the growth of ER-negative PEO23 cells (Figure 3.11). However, a higher concentration of 10^{-5}M caused a significant decrease in cell numbers as compared to control cells. This is similar to the results obtained for the two low ER-status cell lines, PEA1 and PEA2.

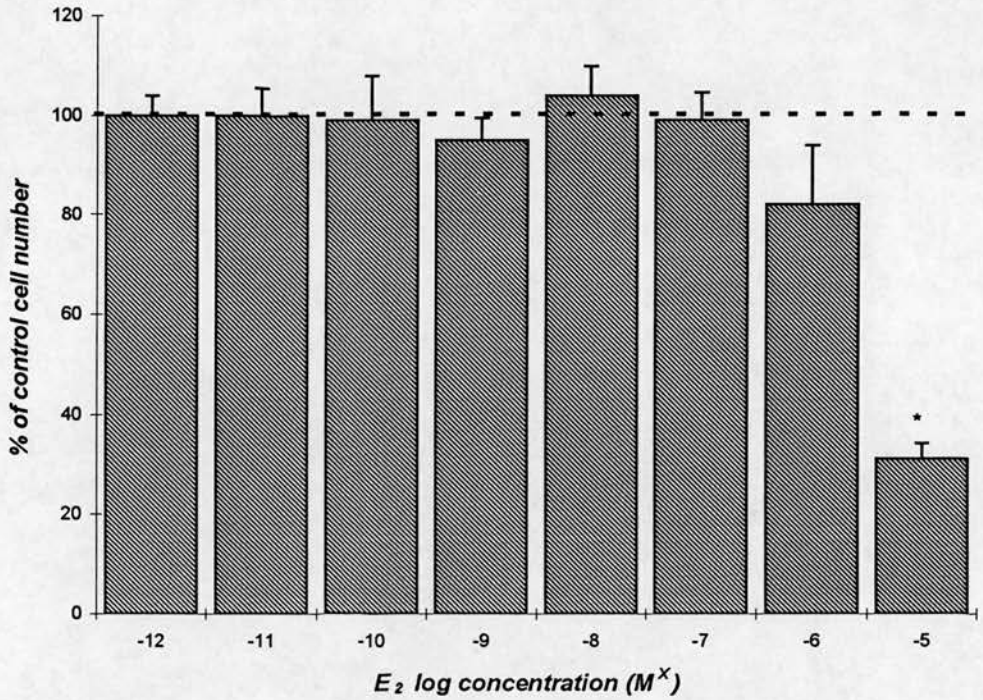


Figure 3.8

Effects of exogenous addition of 17 β-oestradiol (E_2) on PEA1 cell number. Cells were grown in phenol red-free media supplemented with 5% dcsFCS and treated with the concentrations of E_2 indicated over a period of six days. The graph shows the result from one experiment which is typical of at least three identical experiments. Each value represents the mean of quadruplicate samples, each of which was counted in triplicate by Coulter Counter. Error bars denote standard error. *$p<0.05$ for the difference between the control and the group indicated, as evaluated by a Student's *t*-test.

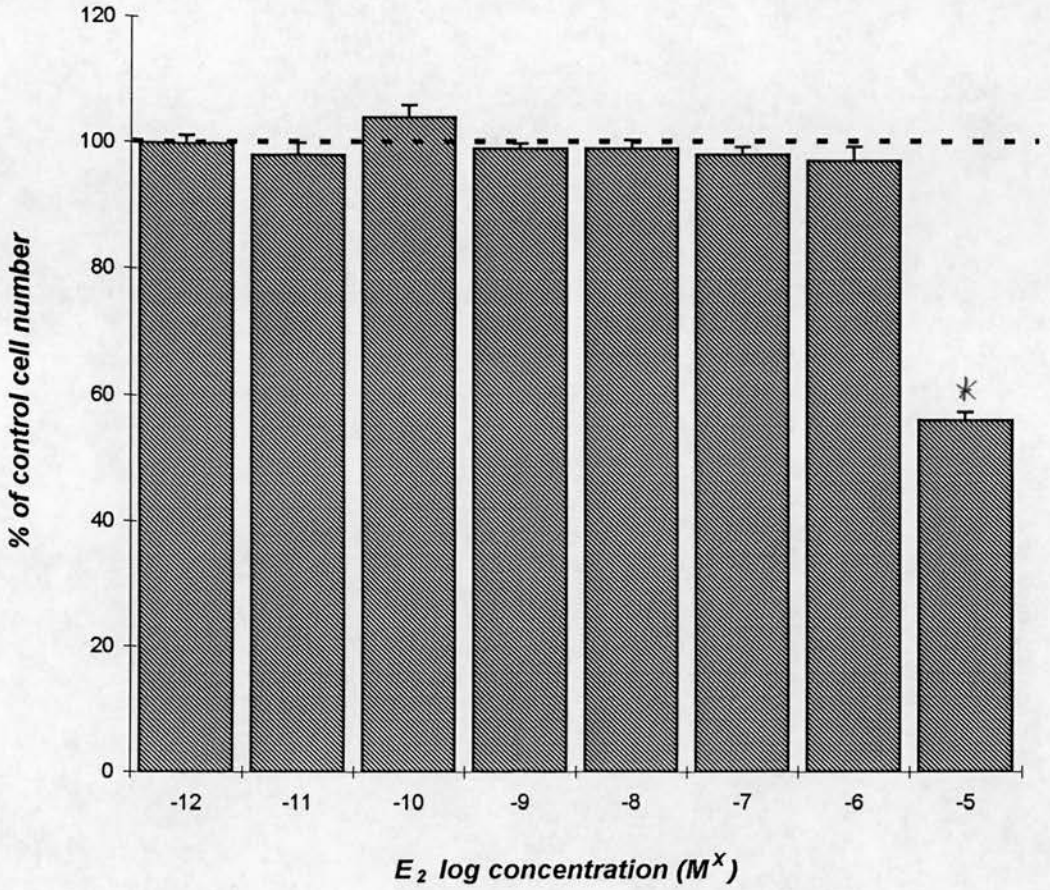


Figure 3.9

Effects of exogenous addition of 17 β-oestradiol (E_2) on PEA2 cell number. Cells were grown in phenol red-free media supplemented with 5% dcsFCS and treated with the concentrations of E_2 indicated over a period of six days. The graph shows the result from one experiment which is typical of at least three identical experiments. Each value represents the mean of quadruplicate samples, each of which was counted in triplicate by Coulter Counter. Error bars denote standard error. *$p<0.05$ for the difference between the control and the group indicated, as evaluated by a Student's *t*-test.

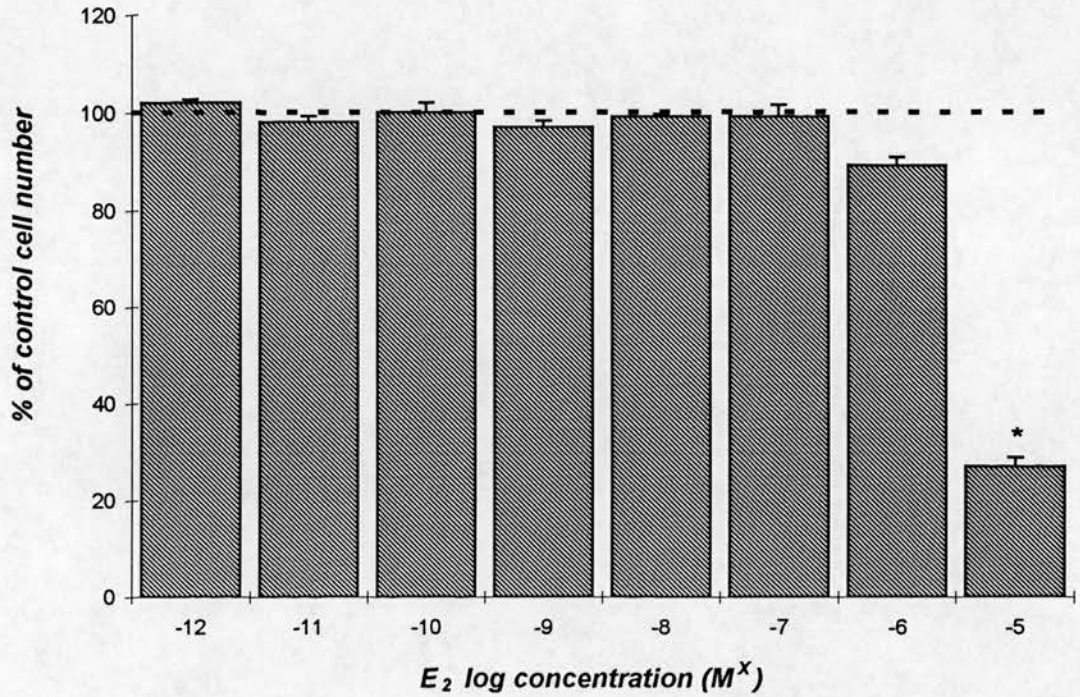


Figure 3.10

Effects of exogenous addition of 17 β-oestradiol (E_2) on PEO16 cell number. Cells were grown in phenol red-free media supplemented with 5% dcsFCS and treated with the concentrations of E_2 indicated over a period of six days. The graph shows the result from one experiment which is typical of at least three identical experiments. Each value represents the mean of quadruplicate samples, each of which was counted in triplicate by Coulter Counter. Error bars denote standard error. *$p<0.05$ for the difference between the control and the group indicated, as evaluated by a Student's *t*-test.

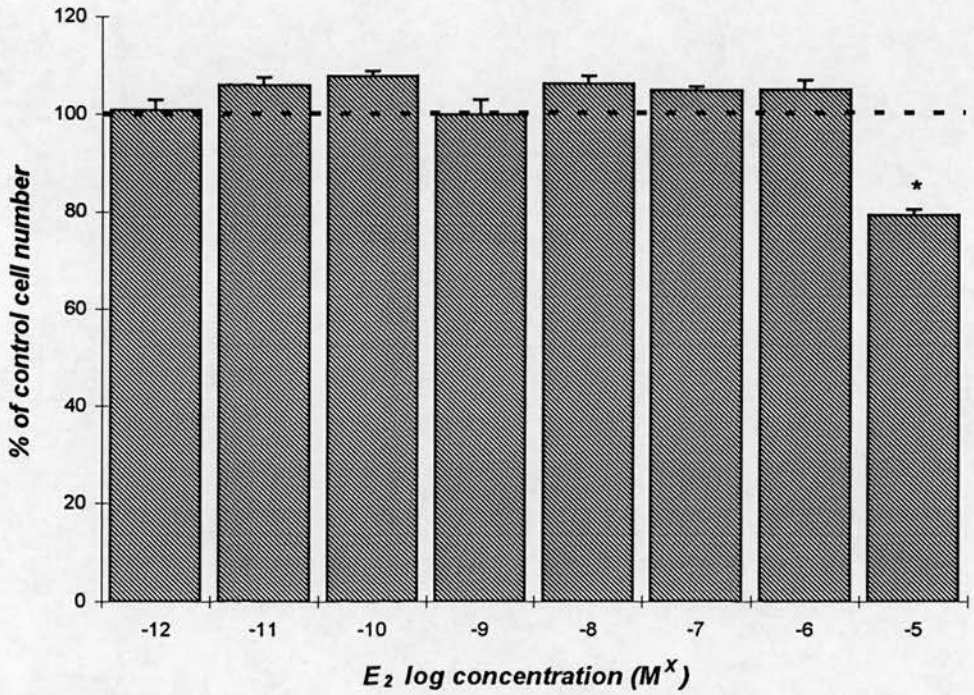


Figure 3.11

Effects of exogenous addition of 17 β-oestradiol (E_2) on PEO23 cell number. Cells were grown in phenol red-free media supplemented with 5% dcsFCS and treated with the concentrations of E_2 indicated over a period of six days. The graph shows the result from one experiment which is typical of at least three identical experiments. Each value represents the mean of quadruplicate samples, each of which was counted in triplicate by Coulter Counter. Error bars denote standard error. *$p<0.05$ for the difference between the control and the group indicated, as evaluated by a Student's *t*-test.

h) PEO1^{CDDP} cell line

Figure 3.12 shows the effects of exogenous addition of 17 β-oestradiol on the platinum resistant PEO1^{CDDP} subline, after six days. The range of concentrations of 17 β-oestradiol (10^{-12}-10^{-5}M) which caused an increase in cell number in the parental PEO1 line above control values, produced a significant decrease in numbers of PEO1^{CDDP} cells, to approximately 40% below that of control cells, the highest concentration (10^{-5}M) producing the greatest inhibition. At lower concentrations of 17 β-oestradiol (10^{-13}-10^{-16} M) a progressive decrease in inhibition was observed with decreasing concentration such that at 10^{-16} M, cell numbers were similar to levels of control cells cultured without oestrogen.

Figure 3.13 shows a more detailed growth response curve for the PEO1^{CDDP} line after treatment with varying concentrations of 17 β-oestradiol. After three days culture an approximate four-fold increase in the number of untreated cells was seen. Cells treated with the range of concentrations of E_2 also showed an increase in number over the three days. However, these numbers were below those seen for the control and decreased in a dose-dependent manner, such that cells treated with the highest concentration of E_2, 10^{-5} M, showed only a 2.6 fold increase in number during this time period.

Following a further three days culture, the number of control cells doubled, whereas a much lower level of growth was seen with E_2-treated cells, this being dose-dependent at the four highest concentrations.

Although PEO1^{CDDP} cells express moderate-high levels of ER (see Table 3.1, p.118) these findings are in direct contrast to the positive growth effects caused by oestrogen in the other cell lines with moderate-high ER levels. It may therefore be more accurate to conclude from these experiments that substantial levels of ER expression in ovarian carcinoma cell lines may indicate oestrogen sensitivity, characterised by a change in growth pattern which may be an increase or a decrease in cell number.

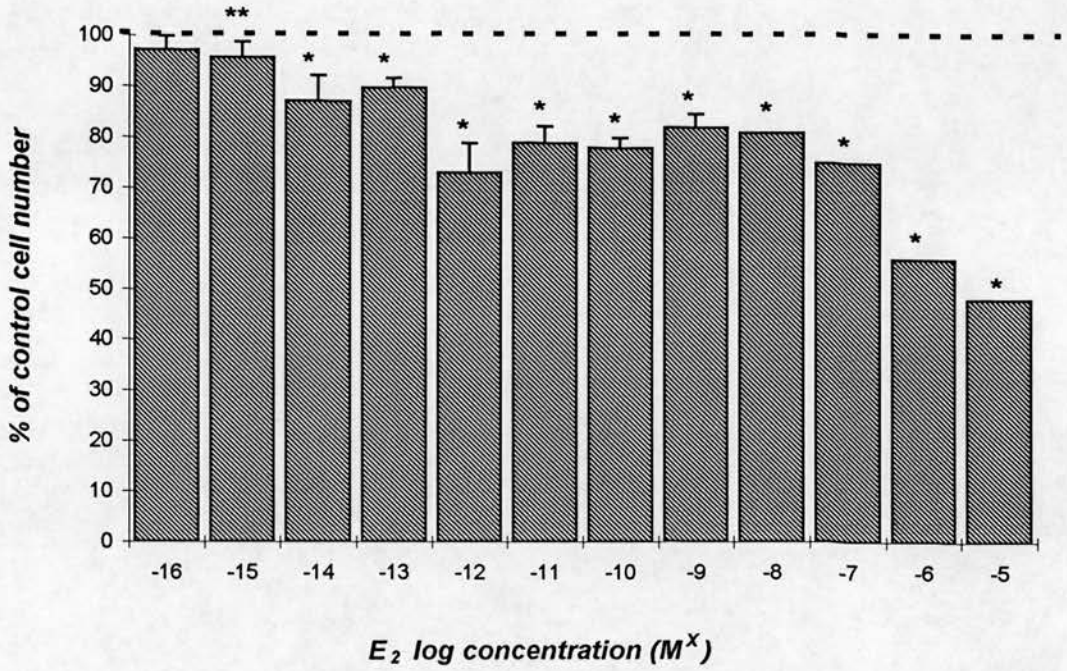


Figure 3.12

Effects of exogenous addition of 17 β-oestradiol (E_2) on $PEO1^{CDDP}$ cell numbers. Cells were grown in phenol red-free media supplemented with 5% dcsFCS and treated with the concentrations of E_2 indicated over a period of six days. The graph shows the result from one experiment which is typical of at least three identical experiments. Each value represents the mean of quadruplicate samples, each of which was counted in triplicate by Coulter Counter. Error bars denote standard error. *$p<0.005$, **$p<0.05$ for the difference between control value and the point indicated, as evaluated by a Student's *t*-test.

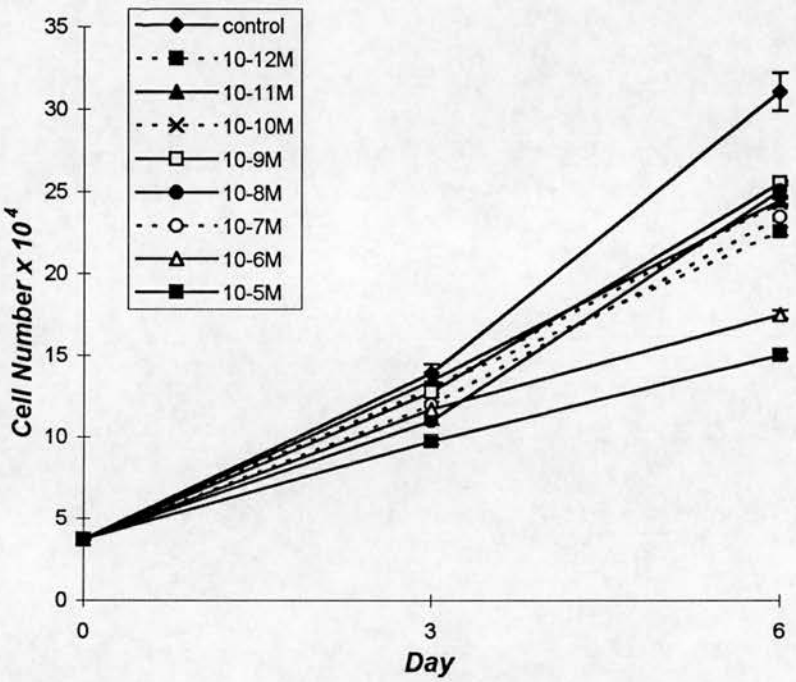


Figure 3.13

Effects of exogenous addition of 17 β-oestradiol (E_2) on the proliferation of $PEO1^{CDDP}$ cells over six days. Cells were grown in phenol red-free media supplemented with 5% dcsFCS and treated with the concentrations of E_2 indicated. The graph shows the result from one experiment which is typical of at least one other identical experiment. Each value represents the mean of quadruplicate samples, each of which was counted in triplicate by Coulter Counter.

3.1.2 Effects of oestrogen on growth *in vivo*

Growth modulation by oestrogen was also examined *in vivo* in two ovarian xenograft cell line models, PEO4 and HOX60 which had previously been established in nude mice. Receptor measurement demonstrated that PEO4 was ER-positive (242 fmol/mg protein) whilst the HOX60 xenograft expressed very low levels of ER (7 fmol/mg protein), nearing the limits of detection. Tumours in the treatment group were exposed to 1.7mg of 17 β-oestradiol in the form of subcutaneously implanted 30-day slow-release pellets, and measured at least once a week. Figures 3.13 and 3.14 show the mean tumour volume in the PEO4 xenograft and the HOX60 xenograft, in untreated and treated animals.

ER-positive PEO4 xenografts without oestrogen supplement increased steadily in volume during the time course. Conversely, there was no apparent change from the initial tumour volume in xenografts which were treated with the 17 β-oestradiol slow-release pellets. Values recorded during the second half of the time course were significantly different to control measurements, (Figure 3.14). This is in sharp contrast to the stimulatory action of oestrogen on proliferation in the *in vitro* PEO4 model.

In mice bearing HOX60 tumours (Figure 3.15), the mean tumour volume of both untreated and treated xenografts increased progressively with time but there was no significant difference in the level of growth between the two groups. This observation correlates well with findings with the *in vitro* ovarian cell line models which show a lack of growth modulation by oestrogen in cells expressing low or negative levels of ER.

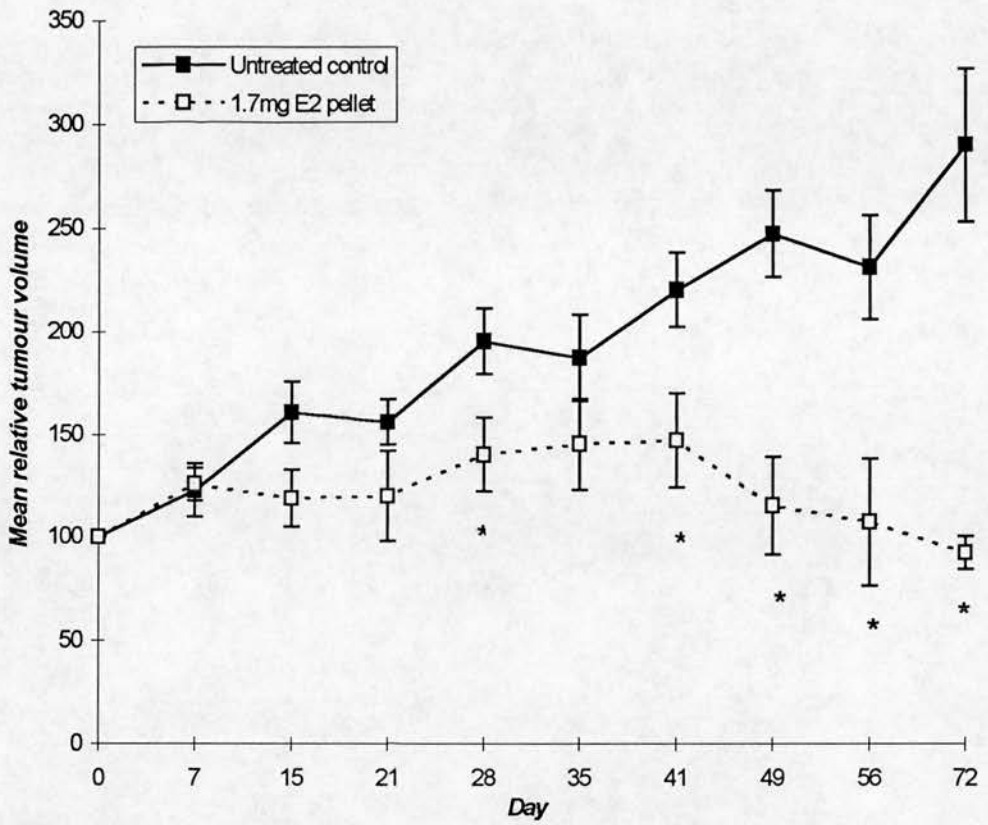


Figure 3.14

Effect of oestrogen on the growth of the PEO4 xenograft. Tumours were established by subcutaneous implant into the flanks of nude mice and exposed to a 1.7 mg 17 β-oestradiol 30 day slow release pellet. Values represent mean relative tumour volume (V_t/V_0) which is the ratio V_t, the volume at the time points indicated, to the initial tumour volume, V_0.(V calculated according to the formula described in the methods). Each point is the mean value for 5-6 mice ± standard error. *$p<0.05$ for the difference between the control and the point indicated, as evaluated by a Student's *t*-test.

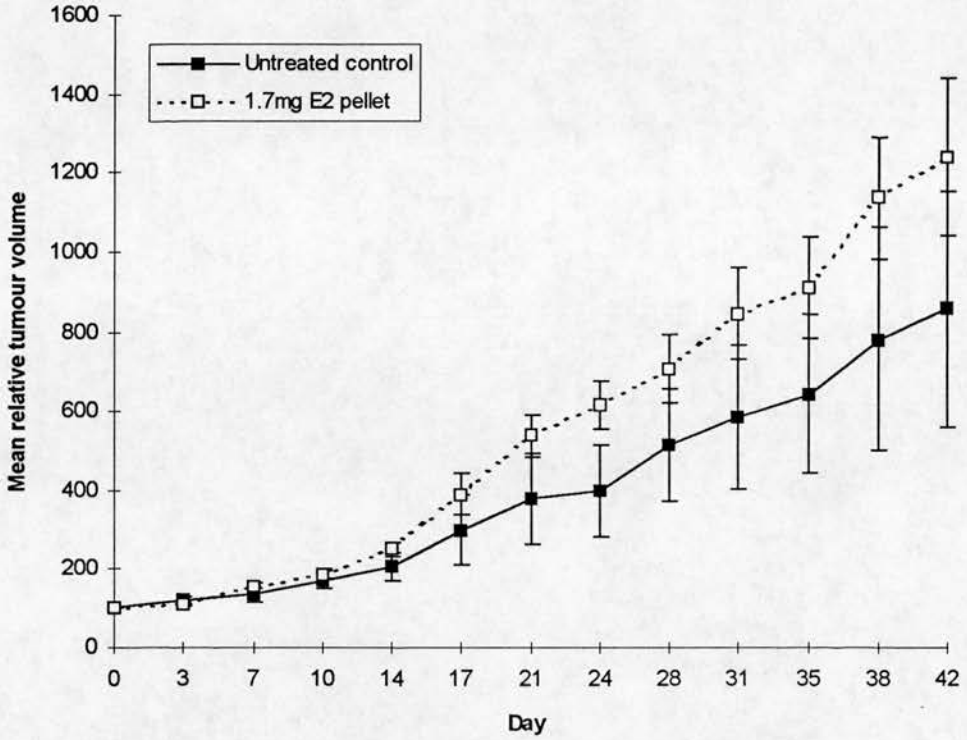


Figure 3.15

Effect of oestrogen on the growth of the HOX60 xenograft. Tumours were established by subcutaneous implant into the flanks of nude mice and exposed to a 1.7mg 17 β-oestradiol 30 day slow release pellet. Values represent relative mean tumour volume (V_t/V_0) which is the ratio of V_t, the volume at the time points indicated, to the initial tumour volume, V_0 (V calculated according to the formula described in the methods). Each point is the mean value for 5-6 mice ± standard error. For each point $p>0.05$ for the difference between control and treated tumours, as measured by a students *t*-test and an ANOVA test.

3.2 Effects of 17 β-oestradiol on the cell cycle

Results from experiments investigating the effects of oestrogen on the growth of ovarian cells, described in the previous section, showed that 17 β-oestradiol caused a growth stimulation in ER-positive lines, in a manner similar to reports in breast cancer cell lines, but had no effect on the growth of cell lines which possessed low or negative levels of ER. However, an exception to this was seen in the cisplatin-resistant derivative of the PEO1 line, $PEO1^{CDDP}$, which showed a marked growth inhibition in response to 17 β-oestradiol after six days despite being ER-positive. It was of interest to perform cell cycle analysis to determine whether oestrogen was affecting the distribution of cells within the cell cycle of the $PEO1^{CDDP}$ cell line. Six cell lines were investigated; PEO1, $PEO1^{CDDP}$, PEO4, PEO14, ZR-75-1 and MDA-MB-231. Cell cycle measurements were taken from cells growing in phenol red-free media supplemented with 5% dcsFCS after 0, 3 and 6 days exposure to 17 β-oestradiol. Figure 3.16 shows representative cell cycle distribution histograms for the PEO4 cell line. Percentages of cells in each phase of the cell cycle were calculated from the size of the peaks, using CellFIT cell cycle software.

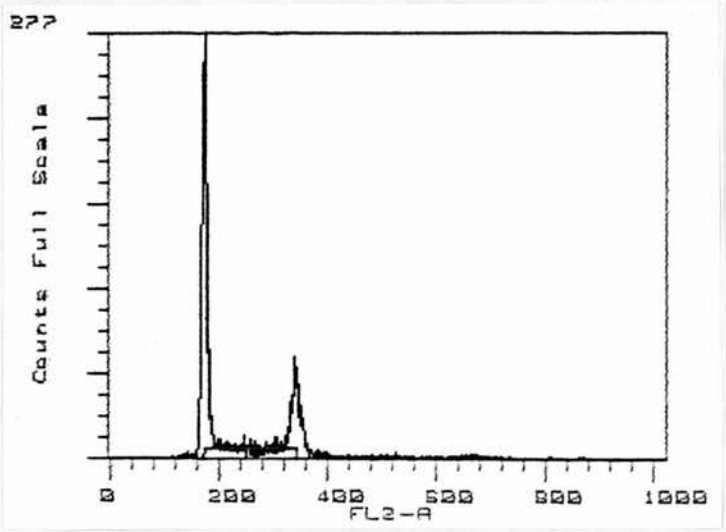
3.2.1 PEO1/ $PEO1^{CDDP}$

Figure 3.17 shows the distributions of PEO1 and $PEO1^{CDDP}$ cells in the cell cycle which had been cultured with or without 17 β-oestradiol (10^{-10}M) for six days. Only minor differences were noted between the two cell lines: on day zero there were slightly more PEO1 cells in G_0/G_1, and slightly more $PEO1^{CDDP}$ cells in S-phase. Over

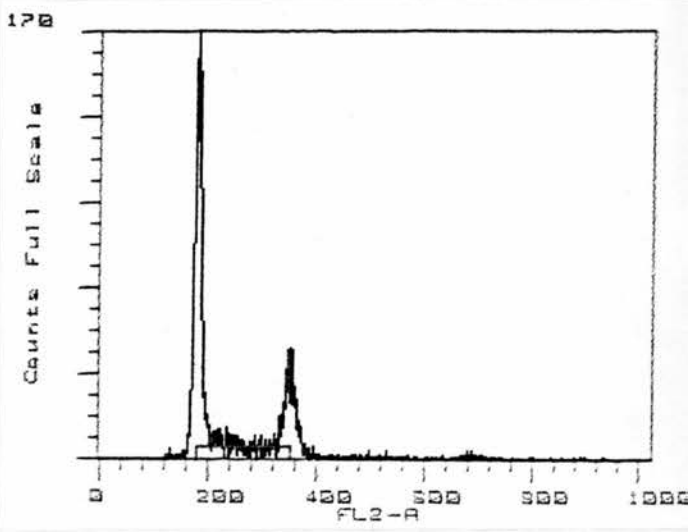
the time point there was a small increase in the number of control PEO1 cells in S-phase, and a decrease in cells in G_2/M. A reduction in the percentage of control cells in G_2/M was also observed with the PEO1^{CDDP} cell line. Only small differences in cell cycle distribution were noted in both cell lines with oestrogen-treated cells. There were slightly higher percentages of oestrogen-treated PEO1 cells in S-phase on day three and day six as compared to untreated cells. Similarly, there was a higher percentage of oestrogen-treated PEO1^{CDDP} cells as compared to control cells in S-phase on day six.

3.2.2 PEO4

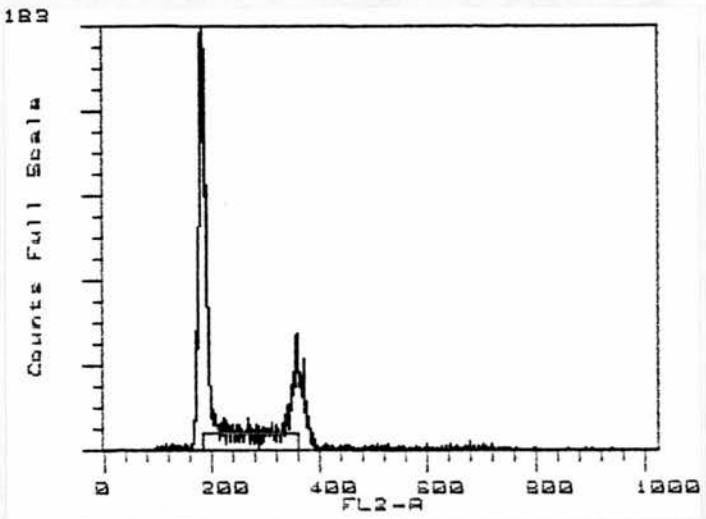
Figure 3.18 shows the cell cycle distribution of untreated and oestrogen-treated PEO4 cells. On day zero, the greatest percentage of cells were in G_0/G_1 (44%), the remaining cells being equally distributed in S-phase and G_2/M. A small increase in the percentage of control cells was noted in G_0/G_1 after three days. This correlated with a small decrease in the percentage of control cells in S-phase and G_2/M. The percentage of untreated cells in G_0/G_1 was lower on day six, with a slight increase in the percentage of cells in S-phase. There were only small differences in the distribution of oestrogen-treated cells as compared to control cells, most noticeable on day six; approximately 10% more oestrogen-treated cells were measured in G_0/G_1, and 10% fewer cells in S-phase.



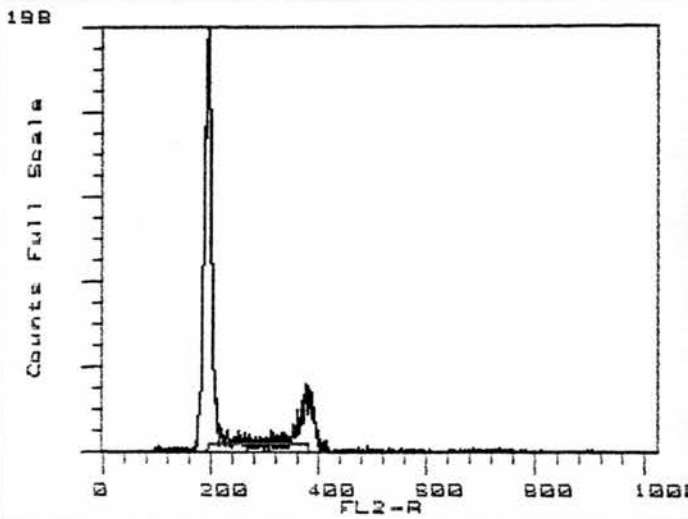
Day 3 Control



Day 3 + E_2



Day 6 Control



Day 6 + E_2

Relative DNA Content

Figure 3.16

Representative cell cycle distribution plots produced using CellFIT Cell cycle analysis software. These figures show the cell cycle distribution of PEO4 cells after culture in phenol red-free medium with or without 10^{-10}M E_2 for three and six days. The x-axis is an arbitrary scale representing DNA content as measured by propidium iodide and the y-axis indicates cell number. The largest peak shown is the G_1 phase of the cell cycle indicating cells which have a diploid (2N) content of DNA, the smaller peak is the G_2/M phase representing cells with twice the DNA content (4N) as cells in G_0/G_1 and the plateau between indicates cells in S-phase which have an intermediate content of DNA.

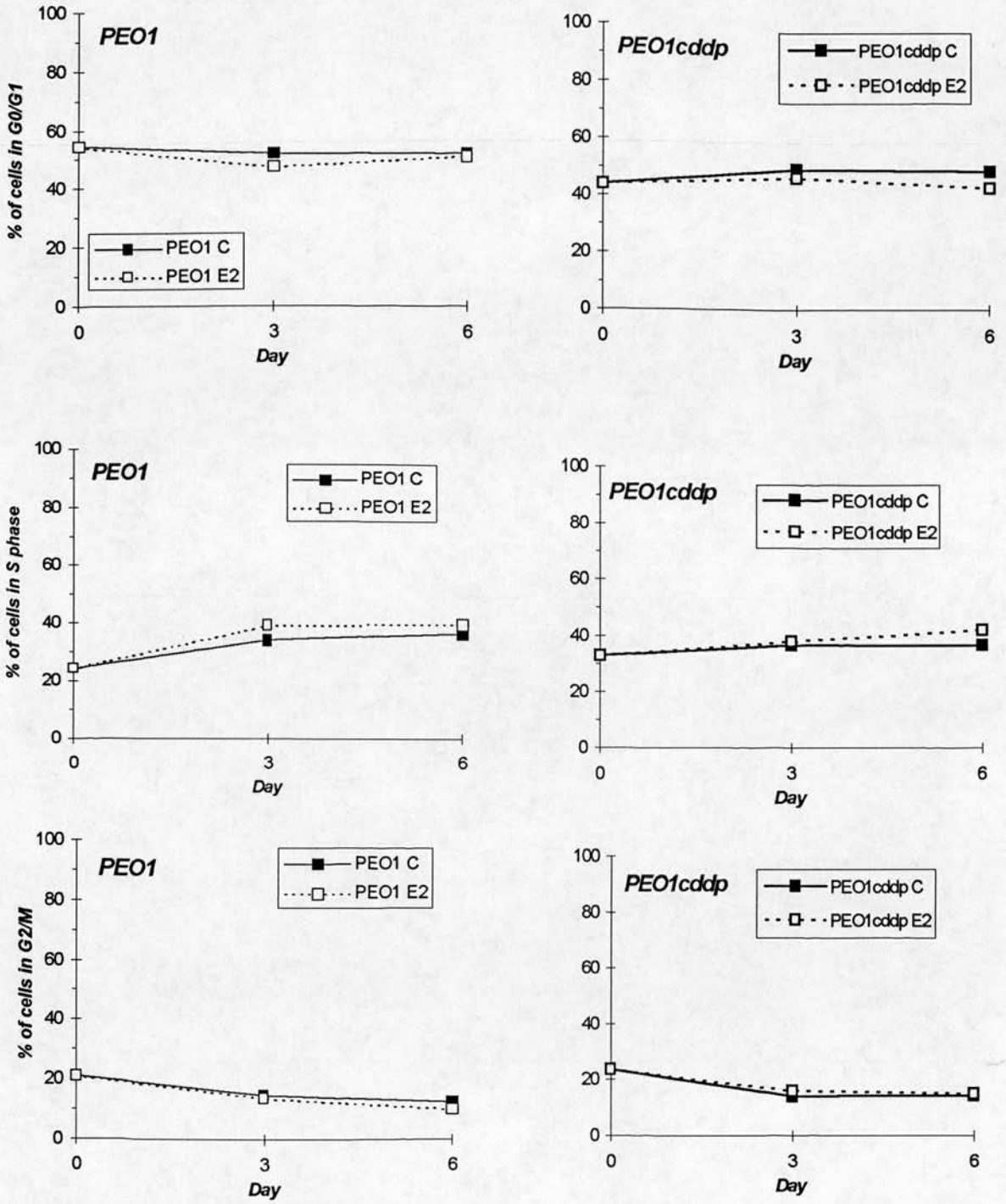


Figure 3.17

The effects of 17 β-oestradiol on the distribution of cells in the cell cycle in the PEO1 and PEO1^{CDDP} cell lines. Cells were cultured for a period of six days either in phenol red-free media containing 5% dcsFCS alone (closed symbols, solid lines) or with the addition of 10^{-10}M 17 β-oestradiol (open symbols, dashed lines). Cell cycle analysis was performed by flow cytometry at the time points indicated. The values shown are from one experiment

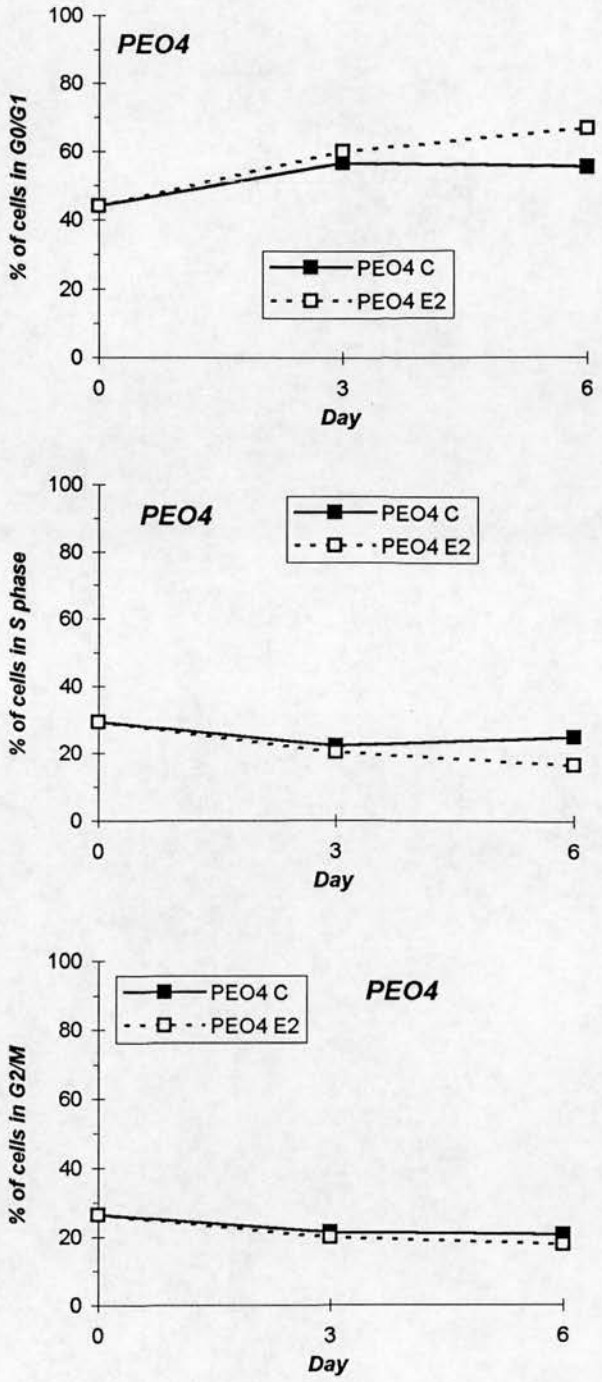


Figure 3.18

The effects of 17 β-oestradiol on the distribution of cells in the cell cycle in the PEO4 cell line. Cells were cultured for a period of six days either in phenol red-free media containing 5% dcsFCS alone (closed symbols, solid lines) or with the addition of 10^{-10}M 17 β-oestradiol (open symbols, dashed lines). Cell cycle analysis was performed by flow cytometry at the time points indicated. The values shown are from one experiment.

3.2.3 PEO14

Figure 3.19 shows the distibutions of ER-negative PEO14 cells in the cell cycle as measured over six days. The highest percentage of cells were in G_0/G_1 on day zero (approximately 66%), with the remainder of cells equally distributed between S-phase and G_2/M. A small increase (5%) in the numbers of control cells in G_0/G_1 was noted after three days, corresponding to a 5% lower percentage of control cells in G_2/M. After six days there were no changes in these levels. The number of PEO14 cells in S-phase remained constant throughout the time course. The distribution of oestrogen-treated cells was similar to control cells.

3.2.4 ZR-75-1

The cell cycle distribution of ZR-75-1 cells is shown in Figure 3.20. The majority of cells (53%) were in G_0/G_1 when measured on day zero, with 37% of cells in G_2/M and 10% in S-phase. A slightly higher number of control cells were in G_0/ G_1 after three days, but this level did not alter when measured after six days. This was associated with a lower percentage of control cells in G_2/M on day three. Numbers of oestrogen-treated cells in G_0/G_1 were similar to control cells after three days, but increased after a further three days so approximately 14% more oestrogen-treated cells were in G_0/G_1 after six days as compared to control cells. This was associated with a slight rise in the percentage of oestrogen-treated cells in S-phase, and a large decrease of cells in G_2/M after six days, so that there were approximately twice the number of oestrogen-treated cells in S-phase as compared to untreated cells after six days. Conversely there were approximately two-thirds more untreated cells in G_2/M.

3.2.5 MDA-MB-231

Figure 3.21 shows the cell cycle distribution for ER-negative MDA-MB-231 breast cells. On day zero, the highest percentage of cells was measured in G_0/G_1 (66%). An increase of cells in G_0/G_1 was noted after three days, and a further small increase was noted on day six. This corresponded to a drop in the percentage of cells in S-phase after three and six days culture. There was no difference in the distribution of oestrogen-treated cells as compared to control cells when measured after three or six days. Overall, the distribution of cells was similar to that observed with the ER-negative PEO14 ovarian cells over the time course.

Summarising the above data: there did not appear to be any major difference in the cell cycle distribution of $PEO1^{CDDP}$ cells as compared to the parental line, PEO1, either with control or oestrogen-treated groups of cells. Far greater numbers of both these cell types, untreated and treated were measured in the S-phase of the cell cycle at the three and six day time points than seen with the other cell lines analysed. In all the cell lines, the majority of cells were found to be in G_1 at the time points measured and in the ER-positive lines, with the exception of the ZR-75-1 cell line, more cells were found to be in the S-phase than G_2/M. There appeared to be equal distributions of ER-negative MDA-MB-231 and PEO14 cells between these two phases. Only in the ZR-75-1 cell line were any large differences noted between the percentages of untreated and oestrogen-treated cells. Twice as many ZR-75-1 cells were measured in S-phase after oestrogen treatment compared to the percentage of control cells, with the converse seen in G_2/M.

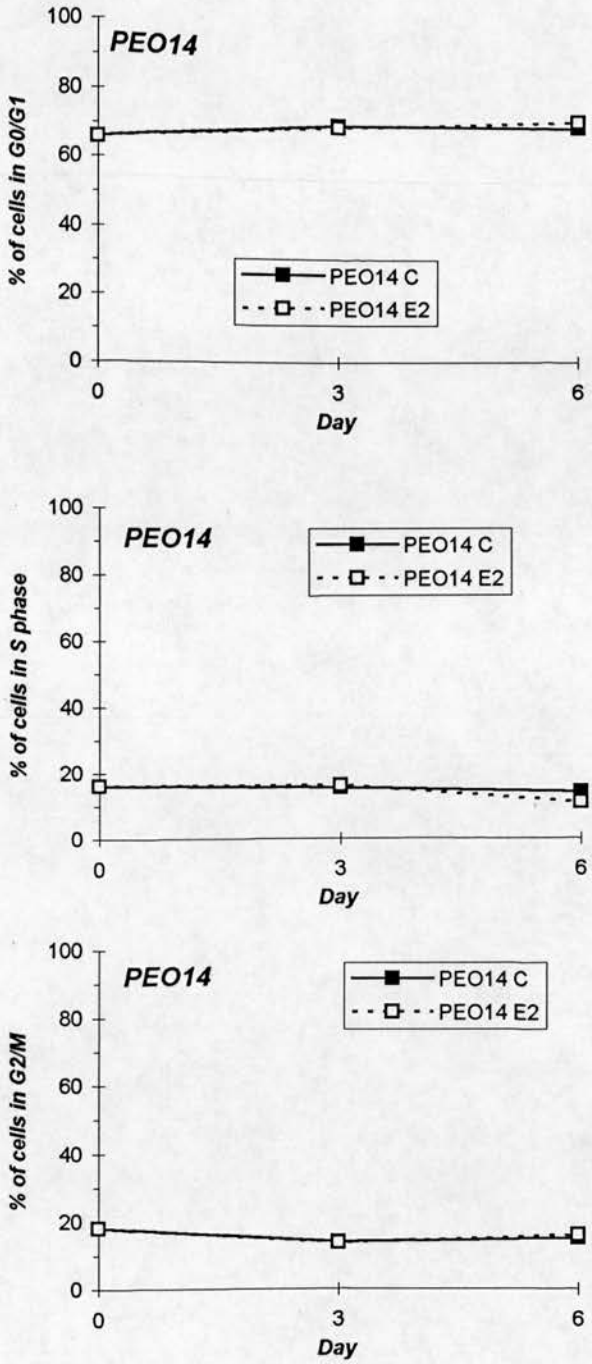


Figure 3.19

The effects of 17 β-oestradiol on the distribution of cells in the cell cycle in the PEO14 cell line. Cells were cultured for a period of six days either in phenol red-free media containing 5% dcsFCS alone (closed symbols, solid lines) or with the addition of 10^{-10}M 17 β-oestradiol (open symbols, dashed lines). Cell cycle analysis was performed by flow cytometry at the time points indicated. The values shown are from one experiment.

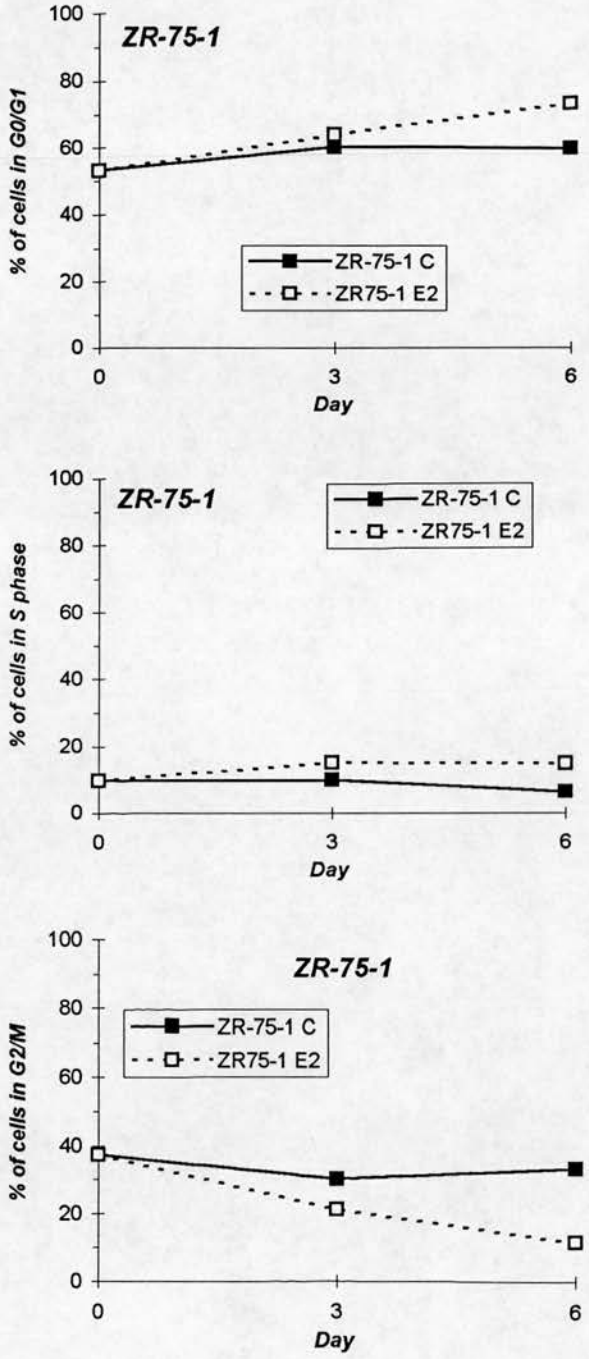


Figure 3.20

The effects of 17 β-oestradiol on the distribution of cells in the cell cycle in the ZR-75-1 cell line. Cells were cultured for a period of six days either in phenol red-free media containing 5% dcsFCS alone (closed symbols, solid lines) or with the addition of 10^{-10}M 17 β-oestradiol (open symbols, dashed lines). Cell cycle analysis was performed by flow cytometry at the time points indicated. The values shown are from one experiment.

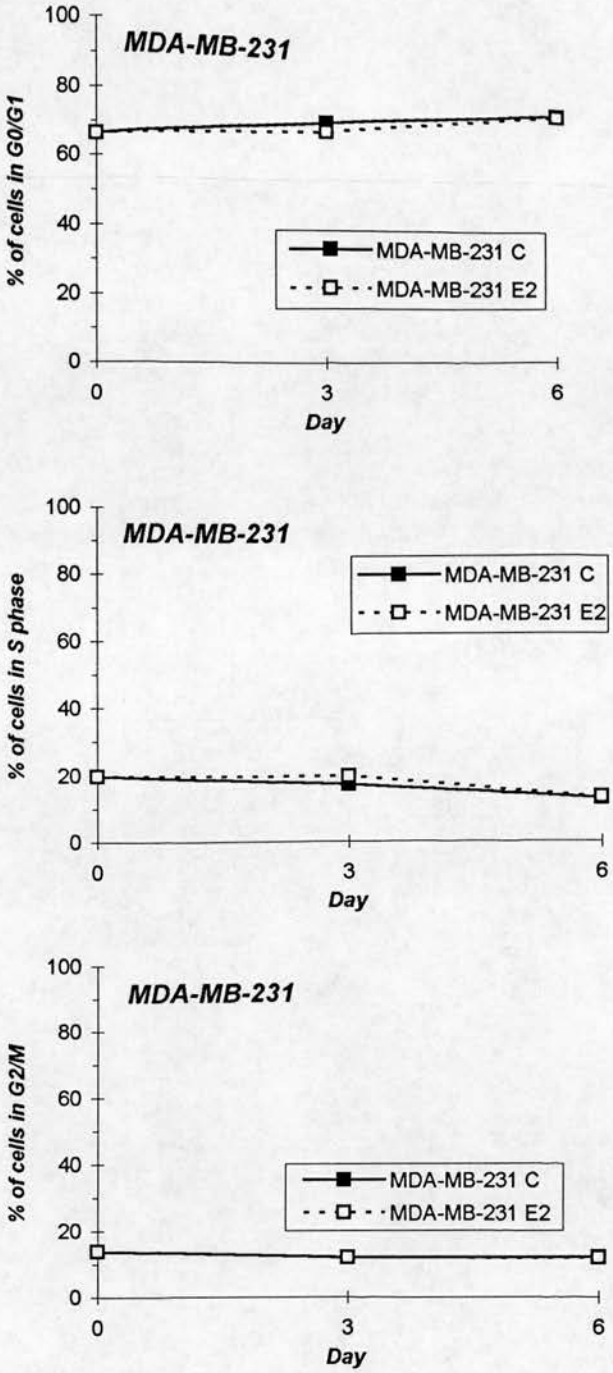


Figure 3.21

The effects of 17 β-oestradiol on the distribution of cells in the cell cycle in the MDA-MB-231 cell line. Cells were cultured for a period of six days either in phenol red-free media containing 5% dcsFCS alone (closed symbols, solid lines) or with the addition of 10^{-10}M 17 β-oestradiol (open symbols, dashed lines). Cell cycle analysis was performed by flow cytometry at the time points indicated. The values shown are from one experiment.

3.3 Effects of oestrogen on oestrogen and progesterone receptor (ER and PR) expression

It has been shown that culture of ER-positive breast cancer cells with oestrogen causes a reduction in the levels of oestrogen receptor levels which are expressed (Horwitz and McGuire, 1976). This is consistent with the notion that E_2 is exerting its effects through the ER. In addition, E_2 increases the expression of another steroid receptor, the progesterone receptor (PR) (Horwitz *et al.*, 1978a). This is one of a number of oestrogen-regulated proteins which may indicate functional ER and therefore have utility as a marker for hormonally-sensitive ER-positive breast tumours. It was therefore of interest to investigate the effects of 17 β-oestradiol on ER and PR expression in the ovarian carcinoma cell lines.

3.3.1 Effect of oestrogen on oestrogen and progesterone receptor expression in ovarian carcinoma cell lines *in vitro*

The ER levels of eight of the ovarian cell lines have been previously recorded (Langdon *et al.*, 1990) using a dextran-coated charcoal adsorption method (Hawkins *et al.*, 1975, 1981). PEO1, PEO4 and PEO6 possess moderate to high concentrations of ER, whereas PEA1 and PEA2 have low levels, and PEO14, PEO23 and PEO16 are ER-negative (Table 2.1). To investigate the effects of 17 β-oestradiol on ER and PR expression, cells were grown in phenol red-free media supplemented with 5% dcsFCS and exposed to a single concentration of 17 β-oestradiol (10^{-10}M) for six days. This concentration provided the maximum growth stimulation in the ER-positive cell lines, PEO1, PEO4 and PEO6 (see section 3.1). Cells were refed after three days and collected by scraping for analysis on day six. Receptor levels

were detected by enzyme immunoassay (EIA) performed by Dr Hawkins in the Department of Surgery, Royal Infirmary, Edinburgh.

(i) Modulation of oestrogen receptor levels

The effect of 17 β-oestradiol on ER expression in the nine ovarian carcinoma cell lines is shown in Figure 3.22, together with the two breast cell lines, ZR-75-1 MDA-MB-231 which were measured for reference. Absolute values, expressed in fmol/mg protein are given in Table 3.1. A cut-off value of 5 fmol/mg protein has been assumed, cells and tissues expressing ER values at or below this background level being considered ER-negative.

Results in Table 3.1 show that of the cell lines investigated, three ovarian cell lines demonstrated significantly ($p<0.05$) lower ER expression after six days exposure to 10^{-10}M 17 β-oestradiol as compared to control cells which were cultured in oestrogen-deprived media; PEO1 cells expressed mean levels of 130 fmol/mg protein when cultured in the absence of oestrogen, but when oestrogen was present, ER expression was substantially lower (approximately three-fold). Levels for ER expression in PEO4 control cells were higher than PEO1 control cells but a similar three-fold level of reduction was noted when cells were cultured in the presence of oestrogen. Likewise, $PEO1^{CDDP}$ cells expressed moderate-high levels of ER expression in the absence of oestrogen, compared to a three-fold reduction in levels when exposed to oestrogen.

One other ovarian cell line, PEO6, expressed moderate-high levels of ER. Untreated PEO6 cells expressed levels of ER at 81 fmol/mg protein. Mean ER values were

approximately 1.5 fold lower in oestrogen-treated PEO6 cells, although this did not achieve significance ($p>0.05$). Low or background levels of ER expression were recorded in the remaining ovarian cell lines, PEA1, PEA2, PEO14, PEO16 and PEO23; and the ER-negative breast cell line, MDA-MB-231, and no significant difference between expression in oestrogen-deprived or treated cells was found.

High levels of ER expression were measured in the ER-positive ZR-75-1 breast cell line, comparable to those found with the PEO4 cell line. Cells cultured without oestrogen had a mean ER value of 271 fmol/mg protein, as compared to 131 fmol/mg protein in cells exposed to oestrogen, a difference of approximately 2-fold, but not statistically significant.

Table 3.1

Effects of 17 β-oestradiol on oestrogen receptor levels in ovarian and breast cancer cell lines. Cells were grown in phenol red-free media, 5% dcsFCS with or without 10^{-10}M 17 β-oestradiol for six days. Receptor levels were measured by enzyme immunoassay and are expressed in fmol/mg protein. Each value is the mean of three separate experiments ± standard error. (*$p<0.05$, representing a significant difference between control and treated cells, as measured by a Student *t*-test.)

ER Concentration (fmol/mg protein)		
	Control	**+ 10^{-10}M E_2**
Ovarian Cancer Cell Lines		
PEO1	129.7 ± 3.7*	47.7 ± 6.1*
PEO4	208.7 ± 21.3*	67.3 ± 6.9*
PEO6	81.1 ± 19.4	55.4 ± 12.9
$PEO1^{CDDP}$	149.4 ± 9.8*	56.5 ± 4.2*
PEA1	18.0 ± 1.4	16.4 ± 0.5
PEA2	7.7 ± 0.9	8.2 ± 0.5
PEO14	5.3 ± 0.9	4.3 ± 0.3
PEO23	8.0 ± 1.0	7.0 ± 1.0
PEO16	7.7 ± 0.9	9.2 ± 0.9
Breast Cancer Cell Lines		
ZR-75-1	271.0 ± 73.6	131.3 ± 22.8
MDA-MB-231	3.5 ± 0.5	4.0 ± 0.0

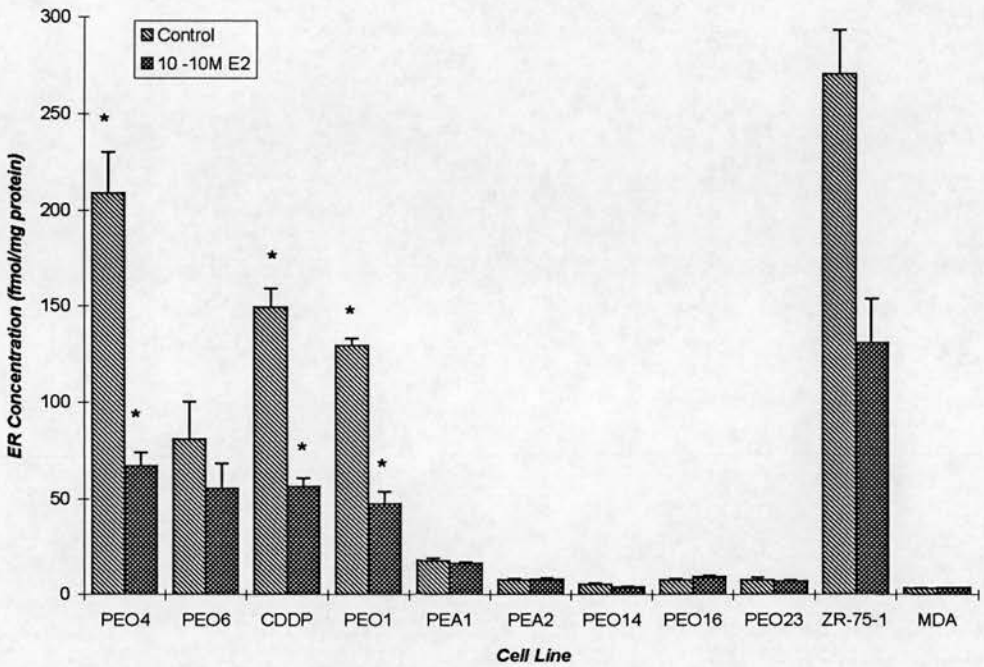


Figure 3.22

Effects of 17 β-oestradiol (E_2) on oestrogen receptor levels in nine ovarian and two breast carcinoma cell lines. Cells were grown in phenol red-free media supplemented with 5% dcsFCS and with or without 10^{-10}M E_2 for a period of six days. ER levels in cell cytosols were measured by enzyme immunoassay and are expressed in fmol/mg protein. Lighter shaded bars depict untreated cells and darker shaded bars represent cells treated with 10^{-10}M E_2. Each value represents the mean of three independent observations and error bars denote standard error. Where there is no apparent error bar, the SEM is small. *$p < 0.05$ for the difference between the control and treated group for the indicated cell line, as evaluated by a Student's *t*-test.

(ii) Modulation of progesterone receptor levels

The effect of 17 β-oestradiol on the level of PR expression in the ovarian and breast cancer cell lines is shown in Figures 3.23 and 3.24; values are recorded in Table 3.2. Low levels of PR expression (2-5 fmol/mg protein) were recorded for all of the ovarian cell lines, and for the ER-negative MDA-MB-231 breast cell line in the absence of oestrogen. In contrast, the ER-positive ZR-75-1 breast cell line expressed substantially higher levels of PR.

Three of the ovarian cell lines, PEO1, PEO4 and PEO6, (high ER levels) showed significant differences ($p<0.05$) in PR expression between the two treatment groups. The biggest differences in PR expression between control and treated cells were observed in the PEO4 and PEO6 cell lines. (see Table 3.2). However, a much smaller difference in PR expression was noted in oestrogen-treated PEO1 cells (5 fmol/mg protein) as compared to the control, these values still being at background levels. The increased level of PR expression seen in both PEO4 and PEO6 in response to oestrogen is comparable to that seen with the ZR-75-1 breast cell line (Table 3.2), which had higher control PR levels (212 fmol/mg protein) but displayed a similar magnitude of increase in PR expression in treated cells (Figure 3.24). PR values for the other ovarian cancer cell lines ($PEO1^{CDDP}$, PEA1, PEA2, PEO14, PEO16 and PEO23) and the MDA-MB-231 breast cancer cell line were at the detection limits of the assay in either treatment group. A small change in PR expression was noted in the MDA-MB-231 cell line but the statistical significance could not be *t*-tested as the SEM of the values for both untreated and treated cells equalled zero.

Table 3.2

Effects of 17 β-oestradiol on progesterone receptor levels in ovarian and breast cancer cell lines. Cells were grown in phenol red-free media, 5% dcsFCS with or without 10^{-10}M 17 β-oestradiol for six days. Receptor levels were measured by enzyme immunoassay and are expressed in fmol/mg protein. Each value is the mean of three separate experiments ± standard error. (*$p<0.05$, for the difference between control and treated values as measured by a Student *t*-test).

PR Concentration (fmol/mg protein)		
	Control	**+ 10^{-10}M E_2**
Ovarian cancer cell lines		
PEO1	3.3 ± 0.3*	4.7 ± 0.3*
PEO4	2.7 ± 0.7*	20.3 ± 6.3*
PEO6	6.3 ± 0.9*	29.0 ± 6.4*
$PEO1^{CDDP}$	3.7 ± 0.3	3.3 ± 0.3
PEA1	3.0 ± 0.0	2.3 ± 0.7
PEA2	2.3 ± 0.3	2.3 ± 0.3
PEO14	2.0 ± 0.0	3.0 ± 0.6
PEO23	7.7 ± 5.3	4.3 ± 2.4
PEO16	4.0 ± 0.6	5.0 ± 0.6
Breast cancer cell lines		
ZR-75-1	212.0 ± 23.1*	920.0 ± 200.4*
MDA-MB-231	3.0 ± 0.0	6.0 ± 0.0

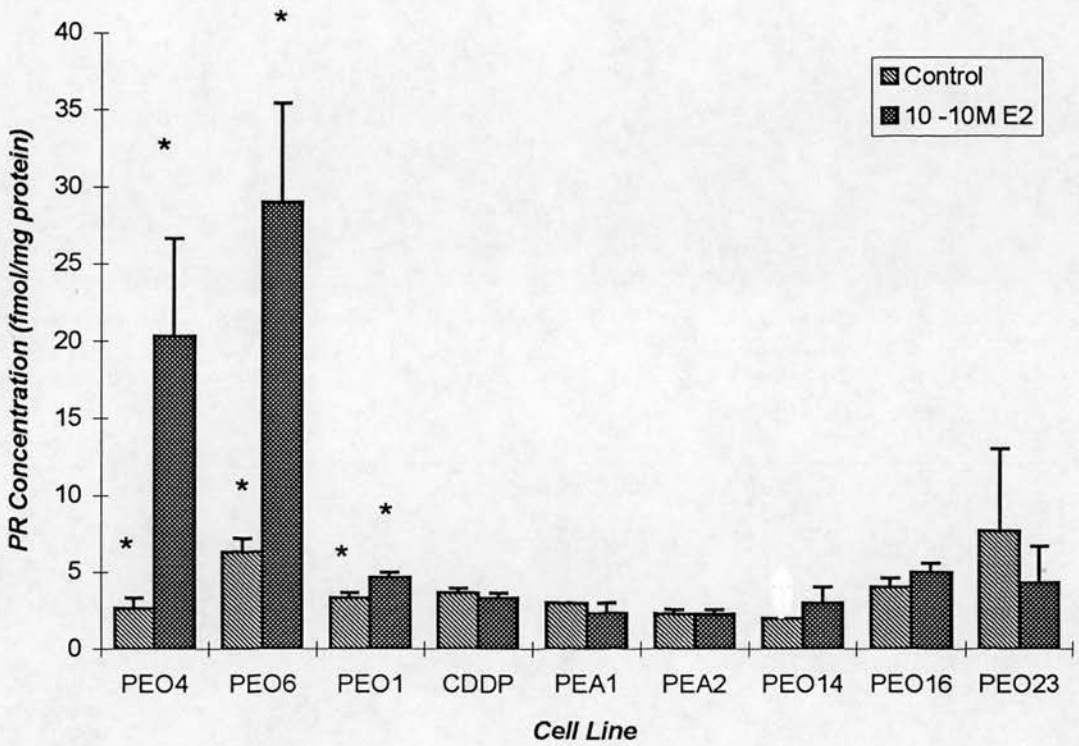


Figure 3.23

Effects of 17 β-oestradiol (E_2) on progesterone receptor levels in nine ovarian carcinoma cell lines. Cells were grown in phenol red-free media supplemented with 5% dcsFCS and with or without 10^{-10}M E_2 for a period of six days. PR levels in cell cytosols were measured by enzyme immunoassay and are expressed in fmol/mg protein. Lighter shaded bars depict untreated cells and darker shaded bars represent cells treated with 10^{-10}M E_2. Each value represents the mean of three independent observations and error bars denote standard error. Where there is no apparent error bar the SEM is small. *$p<0.05$ for the difference between the control and treated group for the indicated cell line, as evaluated by a Student's *t*-test.

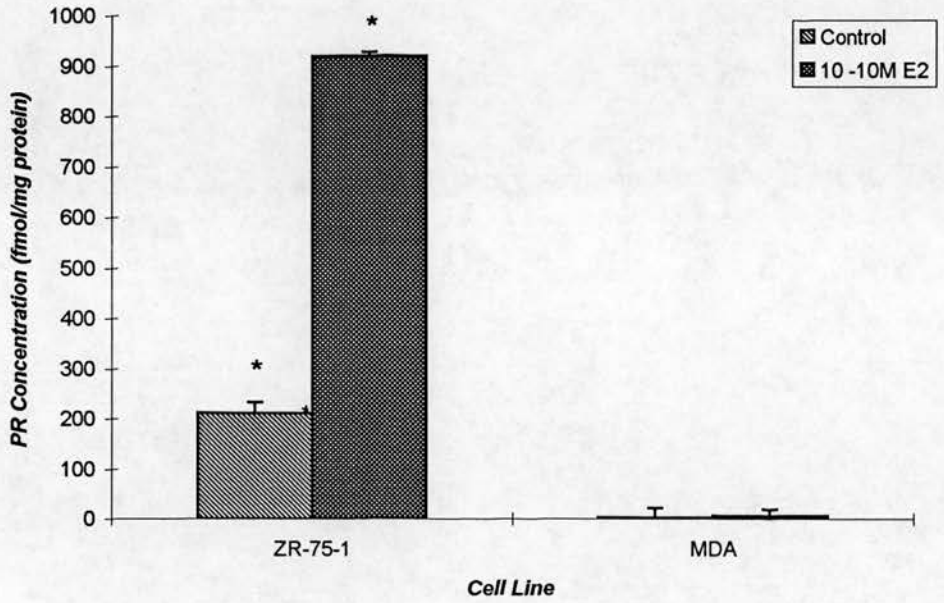


Figure 3.24

Effects of 17 β-oestradiol (E_2) on progesterone receptor levels in two breast carcinoma cell lines. Cells were grown in phenol red-free media supplemented with 5% dcsFCS and with or without 10^{-10}M E_2 for a period of six days. PR levels in cell cytosols were measured by enzyme immunoassay and are expressed in fmol/mg protein. Lighter shaded bars depict untreated cells and darker shaded bars represent cells treated with 10^{-10}M E_2. Values represent the mean of three independent observations, error bars denote standard error. Where there is no error bar the SEM is small. *$p<0.05$ for the difference between the control and treated group for the indicated cell line, as evaluated by a Student's *t*-test.

3.3.2 Effects of oestrogen on oestrogen and progesterone receptor expression in ovarian carcinoma cell lines *in vivo*

The effect of 17 β-oestradiol on ER and PR expression was also investigated *in vivo*, in the two ovarian xenograft models, PEO4 and HOX60. Fragments of xenograft were implanted subcutaneously into the flanks of nude mice and, once established, animals were exposed to a 1.7mg 17 β-oestradiol slow-release pellet planted into the opposite flank of each animal. Control animals did not receive pellets. After 60 days xenografts were removed from both sets of animals and analysed for either ER or PR by enzyme immunoassay.

(i) Modulation of oestrogen receptors

Levels of ER in control and oestrogen-treated ovarian xenografts are given in Table 3.3. The PEO4 xenograft, grown without oestrogen supplement, has high levels of ER (242 ± 41 fmol/mg protein), comparable to the value (209 ± 21 fmol/mg protein) measured in the PEO4 *in vitro* line from which it was derived. When treated with 17 β-oestradiol, ER levels are significantly reduced to approximately 10% of the value (32 ± 2 fmol/mg protein) seen in control animals (see Figure 3.25). In contrast, the HOX60 ovarian xenograft contains borderline levels of ER of 7 fmol/mg protein, (the detection limit of the assay being 5 fmol/mg protein) which are not significantly different from animals receiving oestrogen treatment. These observations parallel the findings seen *in vitro* where ER levels are modulated by oestrogen in cell lines with a moderate-high ER status, but there are no alterations in ER expression in cell lines which contain low levels.

(ii) Modulation of progesterone receptors

Figure 3.26 shows the effect of 17 β-oestradiol on PR expression in the two ovarian xenografts. High PR levels were measured in the ER-positive PEO4 xenograft, but there was only a low level of expression (4 fmol/mg protein) in the ER-negative HOX60 model. There was a marked significant difference in PR expression between oestrogen-treated PEO4 xenografts and those grown without supplement. Mean levels of 87 fmol/mg protein were measured in control animals, but in the treated group the mean value was 776 fmol/mg protein; a nine-fold difference in expression (see Table 3.4). There was no variation in the low levels of PR expression between HOX60 control and treated xenografts.

Table 3.3

Effects of 17 β-oestradiol on oestrogen receptor levels *in vivo* in two ovarian xenografts established in nude mice. Treated xenografts were exposed to a 1.7mg E_2 slow-release pellet and excised after 60 days. Receptor levels were measured by enzyme immunoassay and are expressed in fmol/mg protein. Each value is the mean of three to six separate observations ± standard error. ($p^*<0.05$ for the difference between control and treated xenografts as measured by a Student's *t*-test.)

ER Concentration (fmol/mg protein)		
Ovarian xenograft lines	**Control**	**+ 1.7mg E_2**
PEO4	241.17 ± 41.1*	32.3 ± 1.9*
HOX60	6.7 ± 0.3	8.3 ± 0.3

Table 3.4

Effects of 17 β-oestradiol on progesterone receptor levels *in vivo* in two ovarian xenografts established in nude mice. Treated xenografts were exposed to a 1.7mg E_2 slow-release pellet and excised after 60 days. Receptor levels were measured by enzyme immunoassay and are expressed in fmol/mg protein. Each value is the mean of three to six separate observations ± standard error. ($p^*<0.005$ for the difference between control and treated xenografts as measured by a Student's *t*-test.)

PR Concentration (fmol/mg protein)		
Ovarian xenograft lines	**Control**	**+ 1.7mg E_2**
PEO4	86.8 ± 19.2*	776.3 ± 249.7*
HOX60	3.7 ± 0.3	3.7 ± 0.3

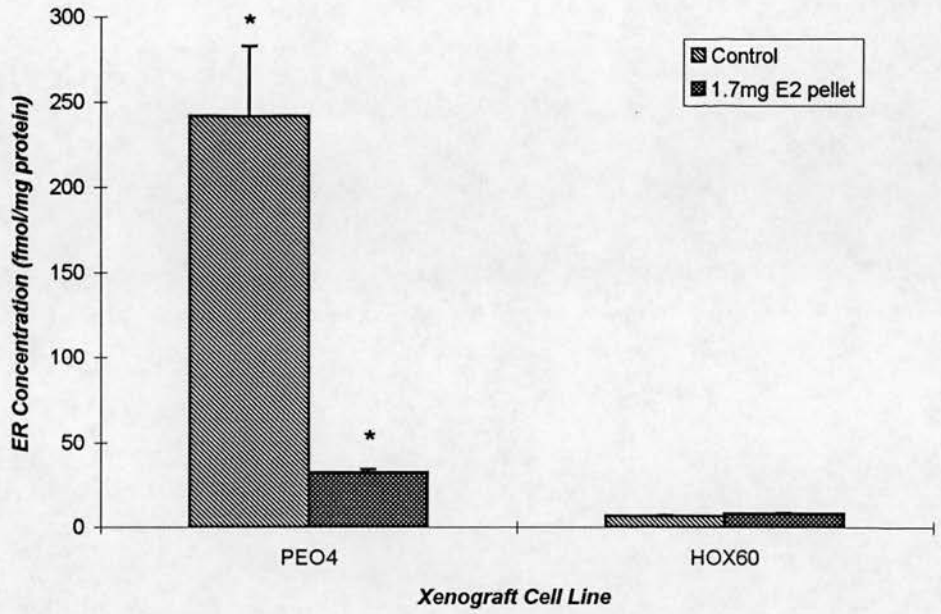


Figure 3.25

Effects of 17 β-oestradiol (E_2) on oestrogen receptor levels in two ovarian xenografts. Tumours were established in nude mice and treated animals exposed to the presence of a 1.7mg E_2 slow release pellet for a period of 60 days. Each value represents the mean of 3-6 independent observations and error bars denote standard error. Lighter shaded bars depict untreated xenografts and darker shaded bars represent xenografts treated with E_2. *$p<0.05$ for the difference between the control and the treated group for the PEO4 xenograft, as evaluated by a Student's *t*-test.

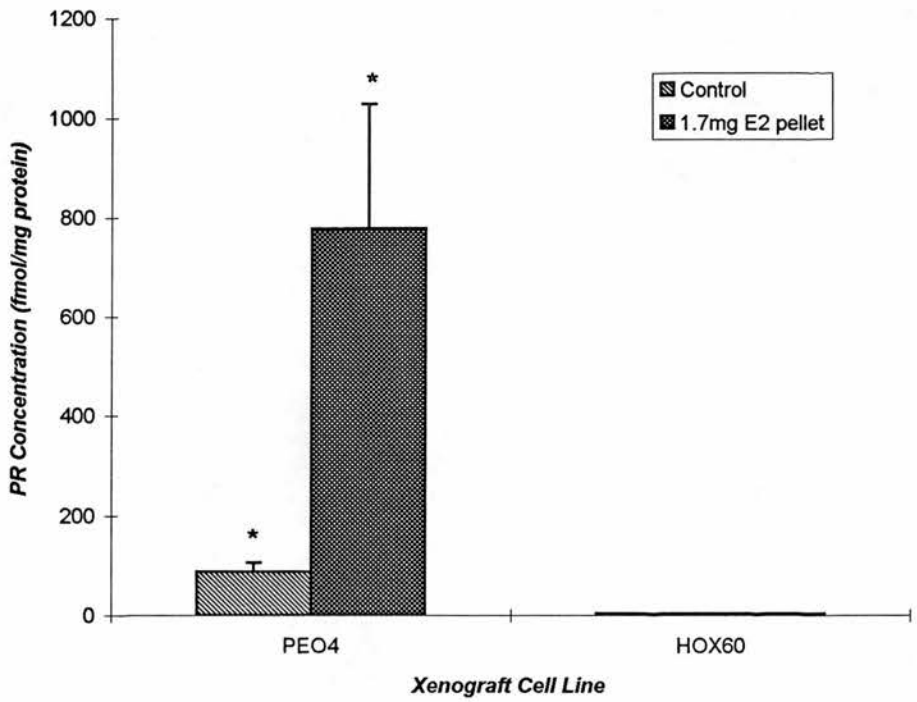


Figure 3.26

Effects of 17 β-oestradiol (E_2) on progesterone receptor levels in two ovarian xenografts. Tumours were established in nude mice and treated animals exposed to the presence of a 1.7mg E_2 slow release pellet for a period of 60 days. Each value represents the mean of 3-6 independent observations and error bars denote standard error. Lighter shaded bars depict untreated xenografts and darker shaded bars represent xenografts treated with E_2. *$p<0.005$ for the difference between the control and treated group for the PEO4, as evaluated by a Student's t-test.

3.4 pS2 expression in ovarian and breast carcinoma cell lines

pS2 is one of a number of oestrogen-regulated proteins which has been cited as having prognostic significance in breast cancer, its expression being found predominantly with ER-positive tumours (Rio *et al.*, 1987, Foekens *et al.*, 1990a, Henry *et al.*, 1991a). In addition, levels of pS2 have been shown to be increased in 17 β-oestradiol-treated MCF-7 breast cells (Masiakowski *et al.*, 1982). However, there have been few studies investigating pS2 expression in ovarian cancer and its role as a marker of hormonal sensitivity is not well defined.

The expression of this protein was therefore investigated in the absence or presence of oestrogen, in several of the ovarian cell lines (PEO1, PEO4, PEA1, PEO14 and $PEO1^{CDDP}$) which express a range of ER levels. Samples were analysed for pS2 expression by flow cytometer after either 3 or 6 days exposure to 10^{-10}M 17 β-oestradiol in phenol red-free media plus 5% dcsFCS, using an in-house protocol which had been optimised for fixation procedures and antibody dilutions. Figure 3.27 shows a typical histogram obtained for ZR-75-1 cells incubated in the presence or absence of oestrogen, showing traces for background staining (in the absence of primary antibody), control and treated cells.

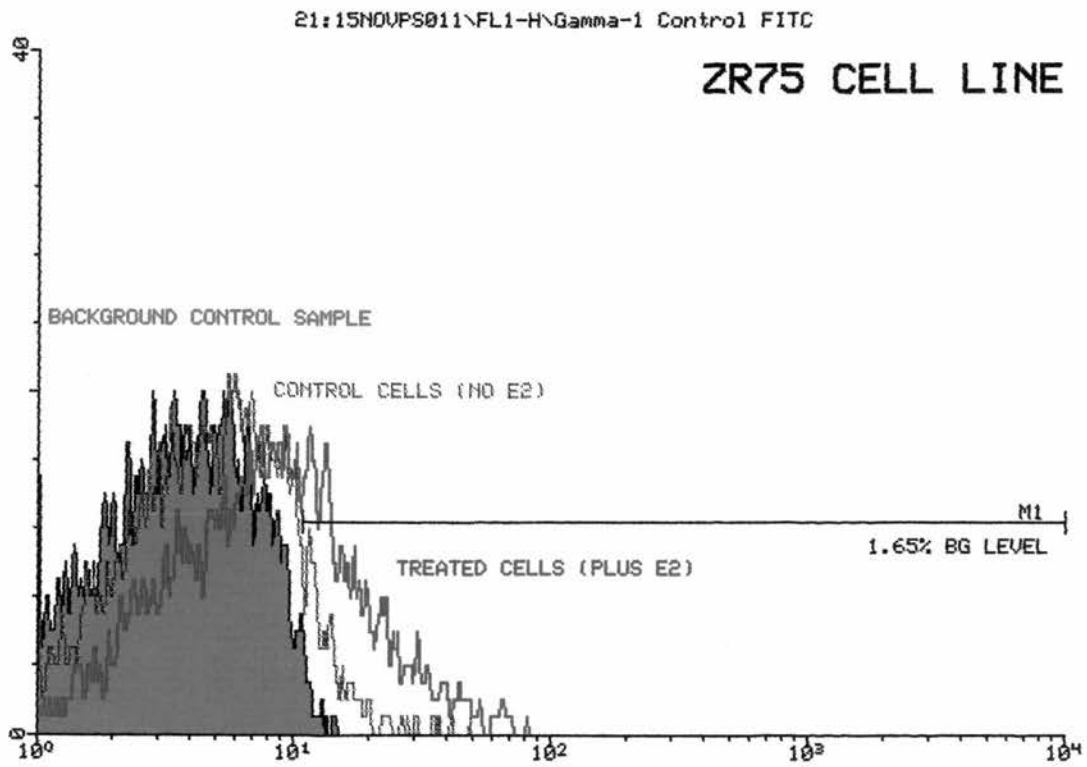


Figure 3.27

Typical histogram obtained for flow cytometric analysis of pS2 expression in ZR-75-1 cells cultured in phenol red-free media supplemented with 5% dcsFCS in the absence or presence of 10^{-10}M E_2 for three days. Plots are shown for background staining (in the absence of the primary antibody, anti-pS2), control cells and oestrogen treated cells. The x-axis shows fluorescence intensity, (4 log decades), and the y-axis cell number (linear scale).

3.4.1 Effect of oestrogen on pS2 expression in ovarian cell lines

The following section describes the results of the effects of oestrogen on pS2 expression, values given being the mean of at least three independent observations. Figure 3.28 shows the intensity of staining for pS2 expression in the five ovarian and two breast cell lines after 6 days, with and without exposure to 10^{-10}M 17 β-oestradiol. These data together with values after three days are summarised in Table 3.5. pS2 expression as measured by staining intensity (mean increase in fluorescence) was at a low level or background levels; i.e. around 1.0 (shown by the dashed line in figure 3.28) at both the time points in all the cell lines which were grown without oestrogen, although a small increase was noted in PEO1 control cells after six days as compared to the day three value. When staining intensity values for oestrogen-treated cells were compared to those grown in the absence of the hormone, the greatest differences were noted in the ZR-75-1 cell line. Levels were significantly raised almost twofold after three days and three-fold by six days exposure. A smaller difference was observed in the PEA1 cells after 3 days exposure to oestrogen compared to cells grown without oestrogen, however the effect was not sustained after six days treatment. Conversely, a slightly lower level of staining intensity was noted in oestrogen-treated PEO1 cells after six days.

pS2 expression was also measured in terms of percentage of cells stained, and Figure 3.29 shows values recorded after six days. A summary of the results is given in Table 3.6. Only a low number of the ovarian cells and the MDA-MB-231 cells stained for pS2, in either control or oestrogen-containing conditions. However, a small significant rise was noted in oestrogen-treated PEA1 cells as compared to the control

after three days; staining in control cells was at background levels but a number of cells treated with oestrogen (5%) showed positive staining. In concordance with results seen with staining intensity in this line, this effect was not sustained following a further three days exposure. There was also a four-fold decrease in the mean level of staining in PEO1 cells exposed to oestrogen for six days as compared to control cells, although again the absolute number of cells staining for pS2 was low. This effect was not noted after three days exposure. This parallels the reduction in staining intensity (Table 3.5) observed with this line.

Expression in control ZR-75-1 breast cells was at a low level, similar to the other cell lines, although there appeared to be a small reduction in the number of positively staining control cells during the time course. Significantly increased numbers of ZR-75-1 cells staining for pS2 expression were measured in the presence of 17 β-oestradiol as compared to levels seen in ZR-75-1 cells growing without the hormone (up to 15-fold higher), the effect being more pronounced with the longer exposure time.

Table 3.5

The effects of 17 β-oestradiol on the expression of pS2 in ovarian and breast cell lines after 3 or 6 days exposure. Values are expressed in terms of increase in mean fluorescence which is a ratio of sample mean fluorescence compared to background values. Thus a value of 1.0 equals background fluorescence. Results shown are the mean of at least three independent observations ± standard error. *p<0.05, **p<0.001 for the difference between control and treated values for the groups indicated, as evaluated by a Student's *t*-test.

	MEAN INCREASE IN FLUORESCENCE (staining intensity) (sample value/background value)			
Cell line	**Day 3**		**Day 6**	
	Control	**E_2**	**Control**	**E_2**
PEO1	1.22 ± 0.10	1.48 ± 0.10	1.65 ± 0.15*	1.17± 0.03*
PEO4	0.99 ± 0.23	1.48 ± 0.10	1.03 ± 0.07	0.98 ± 0.12
PEO6	1.24 ± 0.03	1.28 ± 0.05	1.13 ± 0.20	0.69 ± 0.16
$PEO1^{CDDP}$	0.87 ± 0.30	0.54 ± 0.02	1.21 ± 0.08	1.02 ± 0.10
PEA1	1.01 ± 0.01*	1.43 ± 0.13*	1.20 ± 0.11	1.33 ± 0.10
PEO14	1.16 ± 0.17	1.06 ± 0.11	1.32 ± 0.05	1.33 ± 0.09
ZR-75-1	1.36 ± 0.02**	2.05 ± 0.17**	1.12 ± 0.10**	3.12 ± 0.14**
MDA-MB-231	1.28 ± 0.14	1.28 ± 0.25	1.20 ± 0.10	1.20 ± 0.06

Table 3.6

The effects of 17 β-oestradiol on the expression of pS2 in ovarian and breast cell lines after 3 or 6 days exposure. Values given represent the % of cells stained above background values. Results shown are the mean of at least three independent observations ± standard error and represent values from one experiment typical of at least three similar experiments. *p<0.05, **p<0.01 for the difference between control and treated values for the groups indicated, as evaluated by a Student's *t*-test.

	% OF CELLS STAINED FOR PS2 (sample value-background value)			
Cell line	**Day 3**		**Day 6**	
	Control	**E_2**	**Control**	**E_2**
PEO1	1.87 ± 1.09	7.84 ± 3.32	5.00 ± 0.98**	1.16 ± 0.17**
PEO4	3.79 ± 2.24	8.44 ± 3.09	0.54 ± 0.37	0.99 ± 0.67
PEO6	2.95 ± 0.52	3.27 ± 0.74	1.57 ± 0.60	3.56 ± 0.95
$PEO1^{CDDP}$	0.00	0.00	2.46 ± 0.36	0.98 ± 0.98
PEA1	0.00*	5.45 ± 2.12*	2.54 ± 1.47	5.36 ± 1.33
PEO14	1.16 ± 0.17	1.06 ± 0.11	1.32 ± 0.05	1.33 ± 0.09
ZR-75-1	6.49 ± 1.12**	23.63 ± 3.64**	2.60 ± 0.47**	33.41 ± 2.44**
MDA-MB-231	2.16 ± 1.96	3.58 ± 3.17	0.95 ± 0.42	0.88 ± 0.32

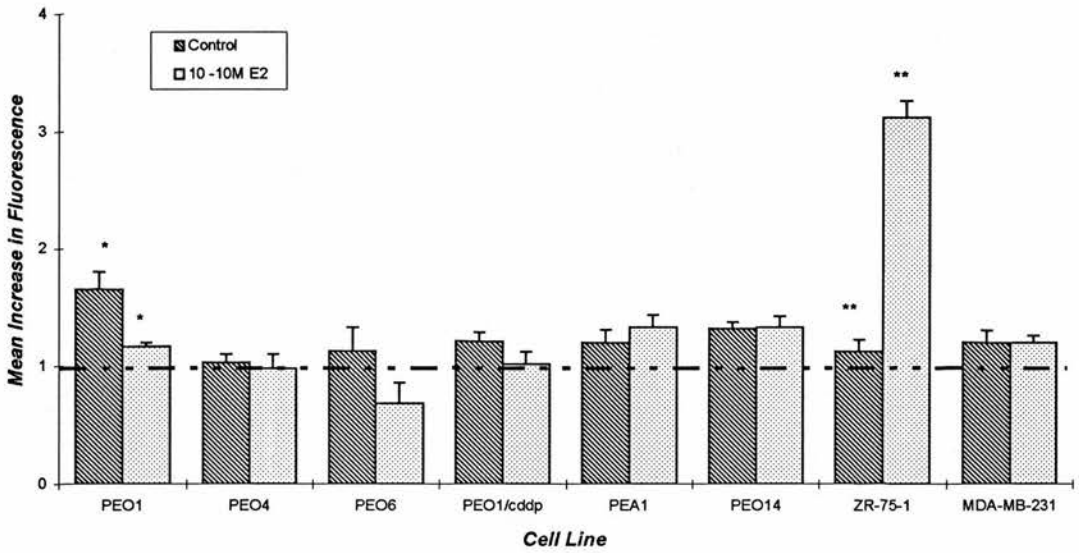


Figure 3.28

Effects of 17 β-oestradiol after six days on the expression of pS2 in ovarian and breast carcinoma cell lines, measured in terms of staining intensity. Cells were grown in phenol red-free media supplemented with 5% dcsFCS. Striped bars represent control cells and shaded bars represent cells treated with 10^{-10}M 17 β-oestradiol. The results shown are the mean of at least three independent observations. The mean increase in fluorescence is a ratio of the sample reading compared to the background, where a value of 1 equals background fluorescence, indicated on the graph by a dashed line. *$p<0.05$, **$p<0.001$ for the difference between control and treated values for the indicated groups, as evaluated by a Student's *t*-test.

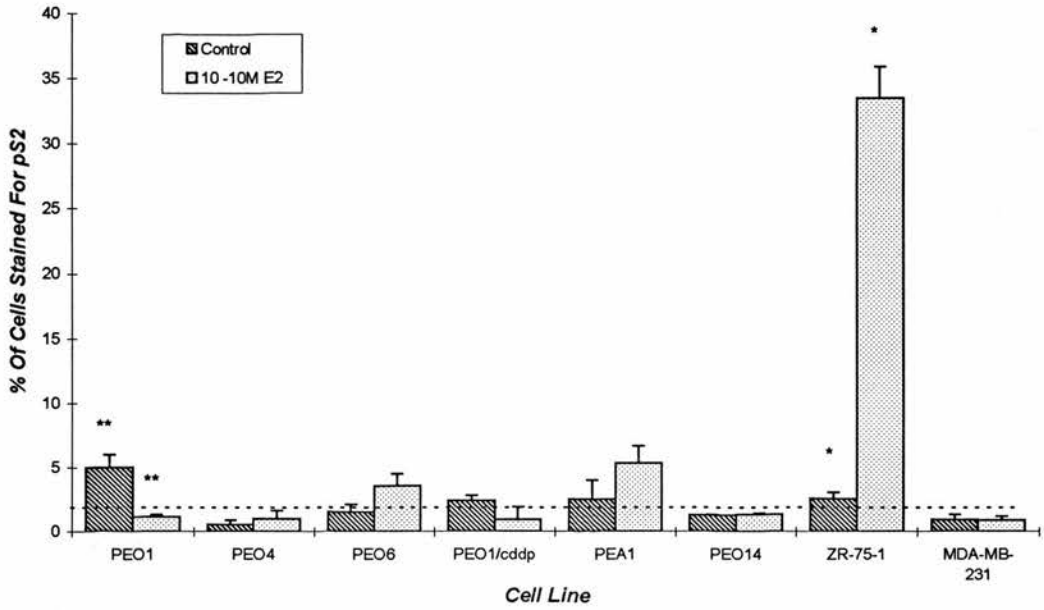


Figure 3.29

Effects of 17 β-oestradiol after six days on the expression of pS2 in ovarian and breast carcinoma cell lines, measured as a percentage of cells positively staining for pS2 above background readings. Cells were grown in phenol red-free media supplemented with 5% dcsFCS. Striped bars represent control cells and shaded bars represent cells treated with 10^{-10}M 17 β-oestradiol. The results shown are the mean of at least three independent observations. *$p<0.05$, **$p<0.01$ for the difference between control and treated values for the indicated groups, as evaluated by a Student's *t*-test.

3.5 HSP27 Expression

HSP27 is an oestrogen-regulated protein in breast cancer in which it is positively associated with the presence of ER (King *et al.*, 1987b). Its expression however, has not been previously reported in ovarian cancer although one study has noted a lack of expression in normal ovary (Ciocca *et al.*, 1983). The expression of this small member of the heat shock family was examined in the series of *in vitro* and *in vivo* ovarian cell lines by an ELISA method developed by Prof. Roger King at the University of Surrey. Initial ELISAs looking at constitutive expression were carried out by Prof. King on cell pellets sent to him for analysis. The effects of oestrogen on HSP27 expression in the cell lines was then examined, incorporating an adapted ELISA method for use in-house.

3.5.1 HSP27 expression *in vitro*

Examination of basal levels of HSP27 in the nine *in vitro* ovarian cell lines was initiated by collecting cell pellets from each of the cell lines grown routinely in phenol red-containing media supplemented with 10% FCS. Cytosols were prepared from these and analysed, the results of which are shown in Figures 3.30 (a) and (b), and summarised in Table 3.7

HSP27 expression was detected by reading the optical densities (OD) at 450nM for each sample dilution, produced by the chromogenic peroxidase substrate, TMB. The intensity of the colour produced, and hence the OD, being proportional to the amount of HSP27 protein present. Final OD readings were calculated by subtracting the

appropriate background values; the OD for the sample minus D5 antibody, and the difference in OD's obtained for a PBS sample incubated with and without D5. Values in Table 3.7 represent the amount of HSP27 (ng/μg of sample cytosolic protein, calculated from the absorbance at 450nM for 1μg protein of sample) divided by the absorbance / ng of recombinant HSP27 protein run in the same assay.

Varying levels of HSP27 expression were observed in the nine ovarian cell lines ranging from background levels in the PEA2 cell line to 3.8 ng/μg in the PEO4 cell line. In general, expression levels appeared to correlate well with the ER status of the cell lines; the highest concentration being noted in the PEO4 cell line, followed by the PEO6 and PEO1 cell lines, which contain moderate to high levels of ER. Lower expression levels were observed in the PEA1, PEA2, PEO16, PEO14 and PEO23 cell lines which express low levels of ER. However, included within this group was the $PEO1^{CDDP}$ cell line which although expresses ER levels similar to the parent line PEO1, contained HSP27 levels similar to PEO16. HSP27 content was also measured in the ER-positive ZR-75-1 breast cell line, which demonstrated almost tenfold higher levels of HSP27 expression than the highest levels found in the ovarian lines (PEO4).

Table 3.7

HSP27 expression in nine ovarian and one breast carcinoma cell lines growing in routine culture conditions; RPMI 1640 containing phenol red and supplemented with 10% FCS. Results are expressed in ng and are calculated from the ratio of the absorbances at 450nM of 1µg of sample cytosolic protein with respect to 1ng of recombinant HSP27. Values show the mean of 3 independent observations ± S.E.M.

Cell Line	HSP27 (ng/µg cytosolic protein)
Cell Lines with moderate/high ER expression	
ZR-75-1	34.8 ± 0.4
PEO4	3.8 ± 0.2
PEO6	2.5 ± 0.6
PEO1	0.9 ± 0.2
$PEO1^{CDDP}$	0.1 ± 0.1
Cell Lines with low or negative ER expression	
PEA1	0.3 ± 0.1
PEA2	0 ± 0
PEO16	0.1 ± 0.1
PEO14	0.4 ± 0.0
PEO23	0.7 ± 0.1

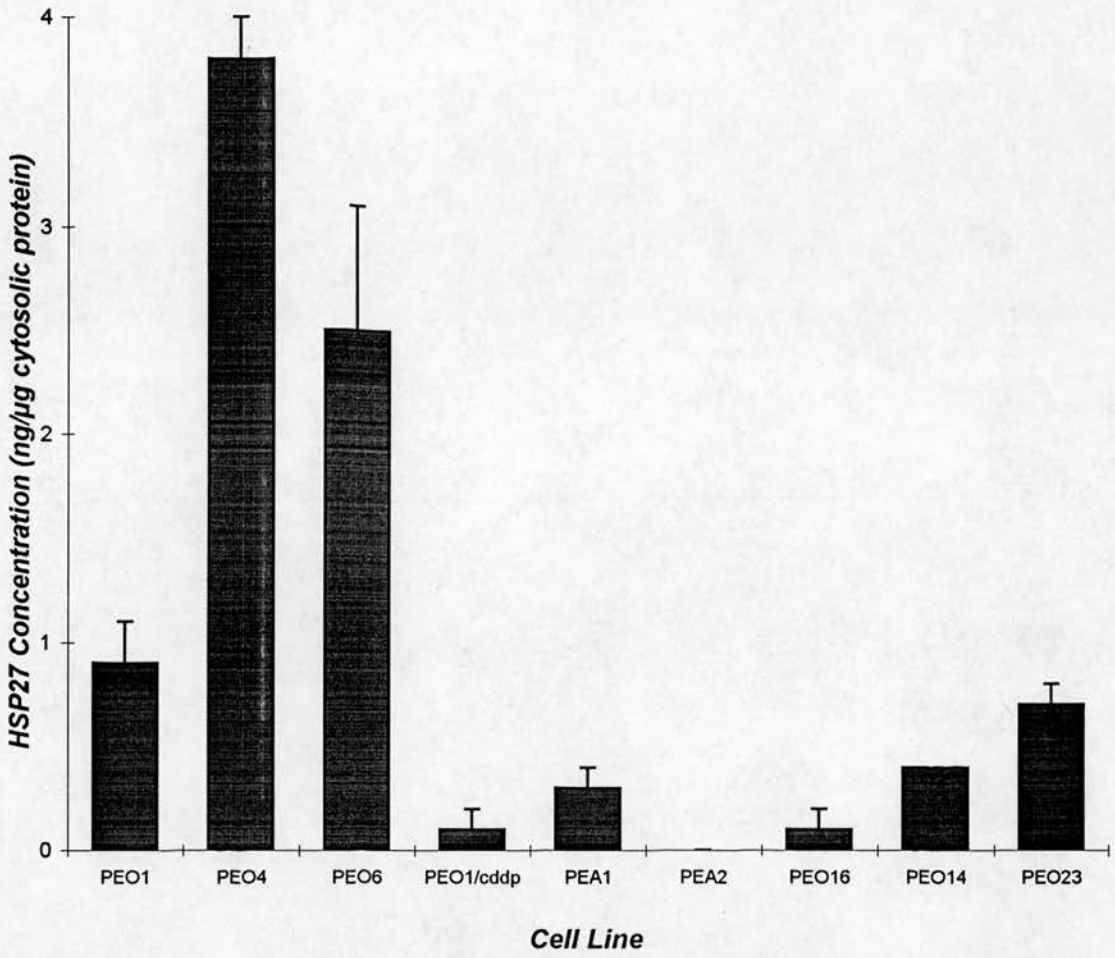


Figure 3.30 (a)

HSP27 expression in nine ovarian carcinoma cell lines. ELISA analysis was performed on cell cytosols, extracted from mid-log phase cells growing in 25cm^2 culture flasks. Bars represent the mean of three separate observations ± SEM and are expressed in ng of HSP27/µg cytosolic protein, calculated from the ratio of the absorbances at 450nM of 1µg protein of sample cytosol with respect to 1ng of recombinant HSP27.

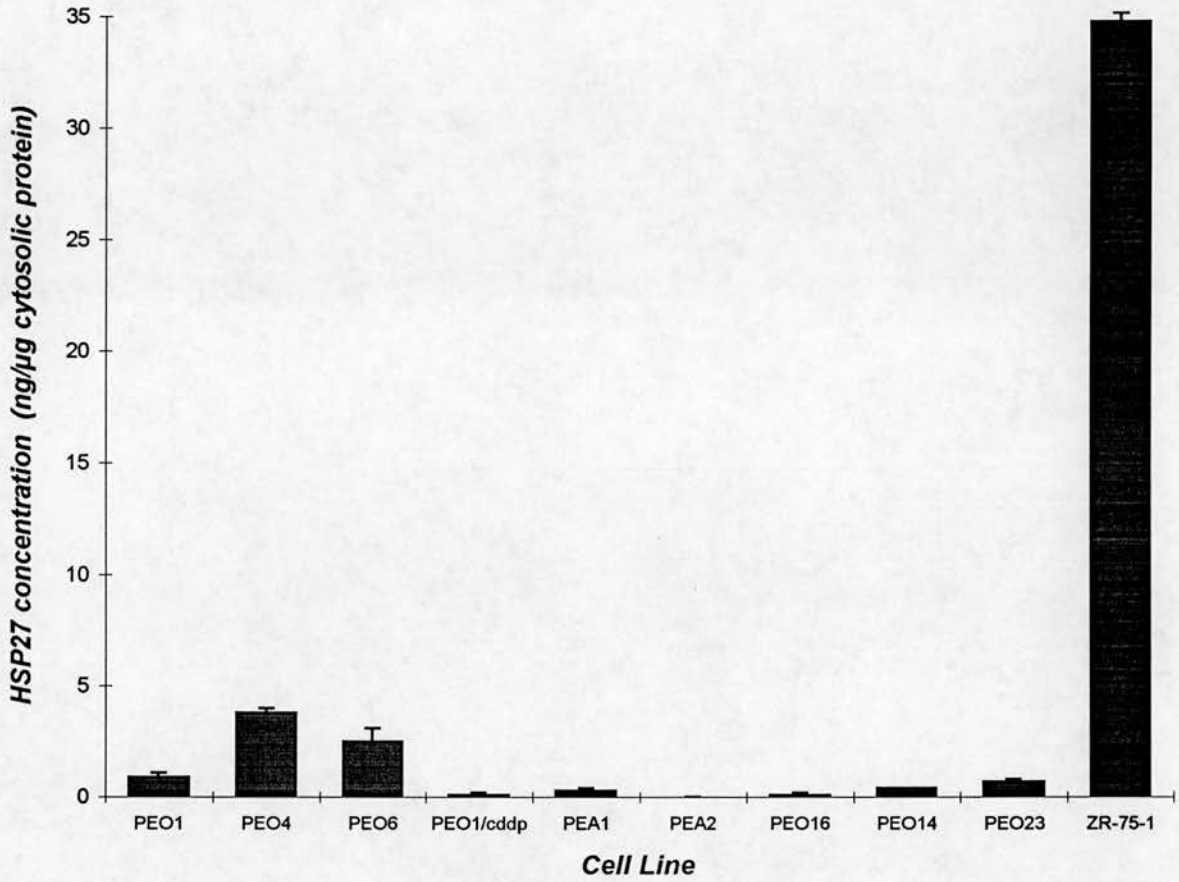


Figure 3.30 (b)

Figure showing HSP27 expression in the ovarian cell lines as compared to that seen in the ZR-75-1 breast cell line.

3.5.2 HSP27 Expression in xenografts

HSP27 expression was also examined in the two ovarian xenograft models, PEO4 and HOX60, and the two breast xenografts, ZR-75-1 and T1068, established, as previously described, in nude (*nu/nu*) mice. The ZR-75-1 model was grown in the presence of additional oestrogen supplement in order to maintain its growth. Results are illustrated in Figure 3.31 and summarised in Table 3.8. Again, consistent with the type of expression observed in the *in vitro* cell lines, the concentration of HSP27 in the xenografts appeared to correlate with their ER status. Thus, higher HSP27 levels were noted in the ER-positive ZR-75-1 and PEO4 xenografts as compared to their ER-negative counterparts, T1068 and HOX60 which expressed very low levels of the heat shock protein. The level of expression which was noted in the ER-positive breast xenograft (60ng/μg), was much higher than that found in the ER-positive ovarian model (1.8ng/μg), also similar to observations *in vitro*. The level of HSP27 expression in the ZR-75-1 *in vivo* model was approximately double that seen in the *in vitro* cell line, and probably reflects the fact that the xenograft is grown in the presence of additional oestrogen supplement, whereas, conversely, the PEO4 xenograft expressed only 50% of the level observed *in vitro*.

Table 3.8

HSP27 expression in two ovarian and two breast carcinoma xenografts established in nude *(nu/nu)* mice. The ZR-75-1 xenograft was established in the presence of a 1.7mg E_2 slow-release pellet. Results are expressed in ng and are calculated from the ratio of the absorbances at 450nM of 1µg protein of sample cytosol with respect to 1 ng of recombinant HSP27. Values show the mean of three experiments ± S.E.M.

Xenograft	HSP27 (ng/µg cytosolic protein)
PEO4	1.8 ± 0.2
HOX60	0.1 ± 0.1
ZR-75-1	60 ± 5.0
T1068	0.1 ± 0.1

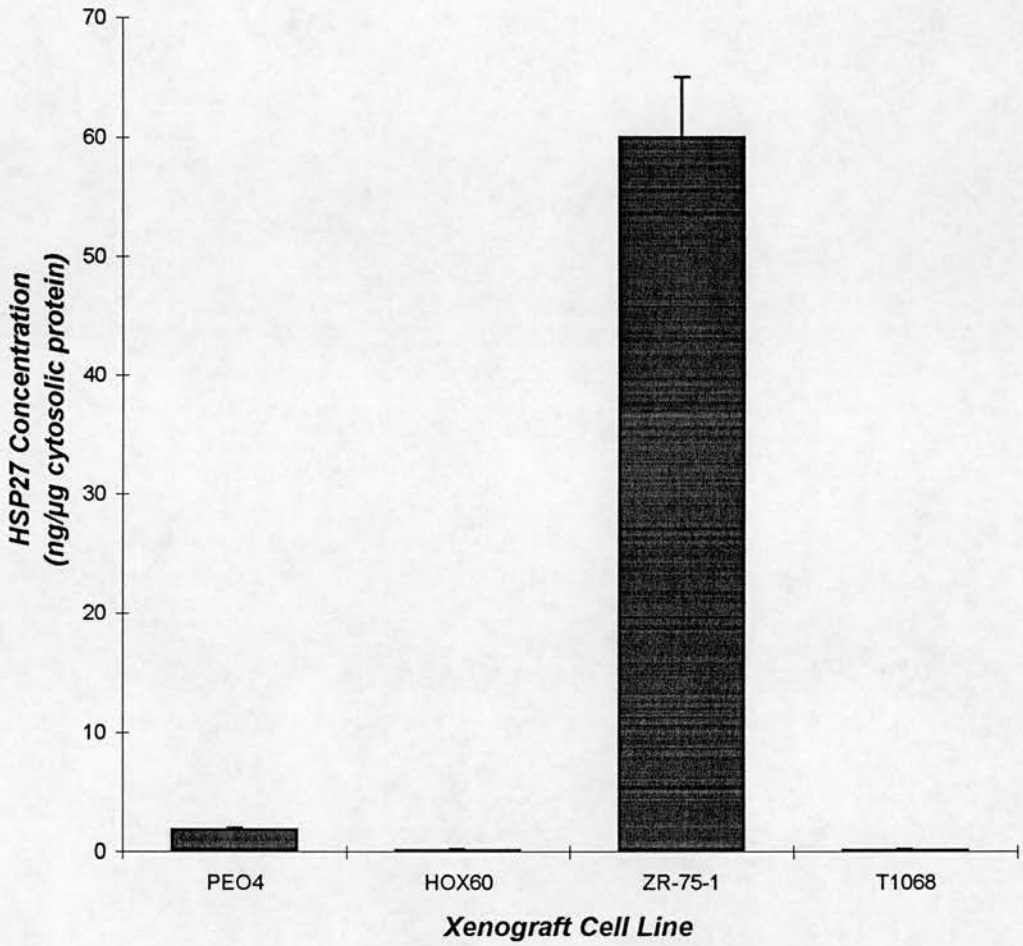


Figure 3.31

HSP27 expression in two ovarian and two breast carcinoma xenografts. ELISA analysis was performed on cell cytosols, extracted from tumours established in nude (*nu/nu*) mice. Bars represent the mean of three separate observations ± SEM and are expressed in ng of HSP27/µg cytosolic protein, calculated from the ratio of the absorbances at 450nM of 1µg protein of sample cytosol with respect to 1ng of recombinant HSP27.

3.5.3 Effects of oestrogen on HSP27 expression *in vitro*

An adapted ELISA method was used to investigate the modulation of HSP27 expression by 17 β-oestradiol in the ovarian *in vitro* and *in vivo* cell models.

(i) Validation of method

Before the ELISA was run routinely to detect HSP27 modulation, several aspects of the method were investigated to determine optimum conditions.

a) Substrate optimisation

In previous studies, both *o*-phenylene diamine hydrochloride (OPD) and tetramethylbenzidine (TMB) have been utilised as peroxidase substrates for the HSP27 ELISA depending on the type of sensitivity required. TMB has been reported to be the more sensitive of the two assay systems and more appropriate for use with cell lines which may express lower levels of HSP27, as opposed to tumour material which may express a large range of HSP27 concentrations (R. King, personal communication). To determine which would be the one of choice for detecting modulation in the ovarian cell lines, titrations of recombinant HSP27 protein (r-HSP27) and cytosols prepared from human myometrial cells were run in an ELISA with both substrates. Human myometrium expresses a high level of HSP27 (Ciocca *et al.*, 1983), and has previously been used as a standard in ELISA's before the availability of the recombinant protein. Studies with this ELISA had suggested protein concentrations between 20 and 0.5 μg /ml protein would be a suitable range for the myometrial standard. Routinely cultured ZR-75-1 and MDA-MB-231 cells,

were also examined, at a range of dilutions; 5, 0.5, 0.05μg /protein/ml, covering a range suggested by previous work (Dunn *et al*,. 1993).

Figure 3.32 gives the results for the recombinant HSP27, myometrial and breast samples which were incubated with both substrates for 10 minutes in the dark at room temperature.

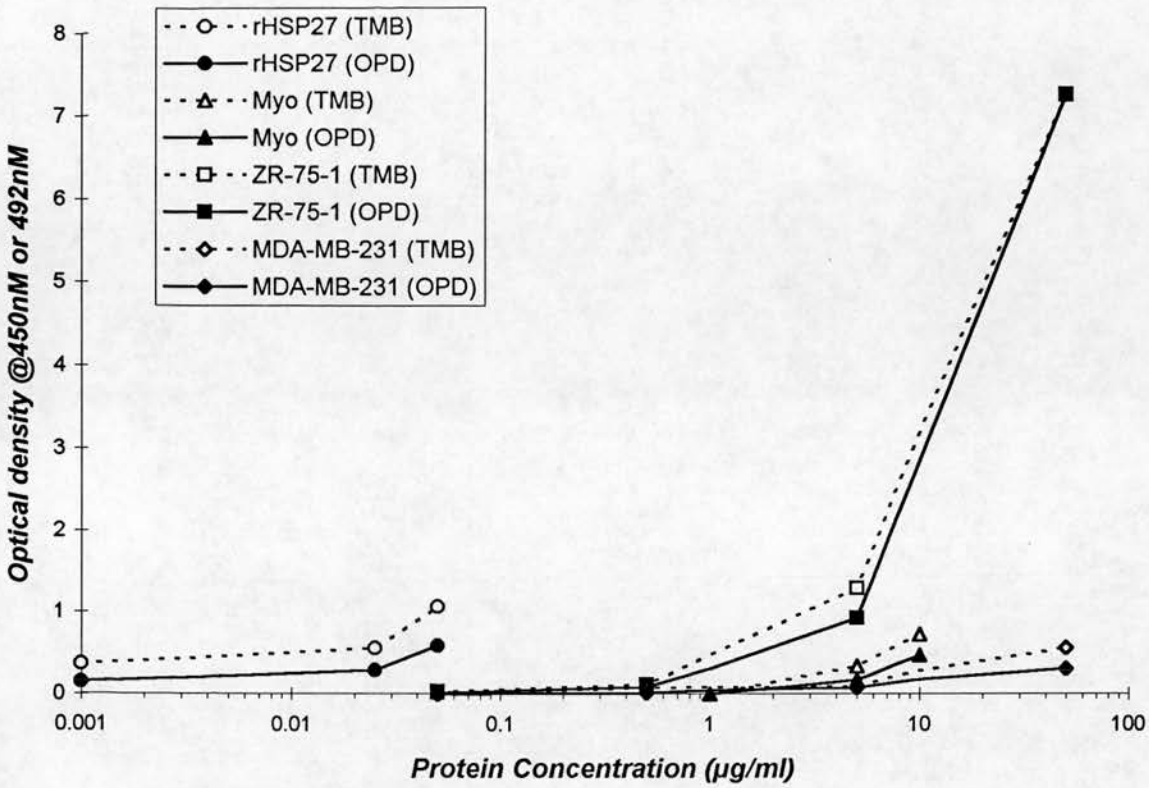


Figure 3.32

Evaluation of two different peroxidase substrates, TMB and OPD, in the detection of HSP27 expression by ELISA. Values represent the optical densities at the appropriate wavelengths of the two substrates after 10 minutes incubation at room temperature in the dark (TMB; 450nM, OPD; 492nM). Results are shown from one experiment which was repeated again with similar findings.

The results shown in Figure 3.32 are final OD values resulting from the subtraction of background readings. Background OD's were obtained from cytosolic samples in the absence of the primary antibody D5, and PBS samples +/- D5. From these results it appeared that there was little difference in the two substrates, although slightly higher OD readings were obtained using the TMB substrate, possibly suggesting that it was more sensitive to the levels of recombinant HSP27 protein, (r-HSP27), and HSP27 expression in myometrium and breast samples. TMB was therefore incorporated as the substrate in further studies as it might be more likely to detect any small changes in HSP27 expression in the cell lines due to modulation by oestrogen.

b) Standard optimisation

Previous studies have incorporated human myometrium as a standard in HSP27 assays. However, as the recombinant protein has recently become available it seemed appropriate to include this in these studies, to provide a reliable constant value. Titrations of both standards were evaluated initially using the OPD peroxidase substrate and the results from a single experiment are shown in Figure 3.33. The concentrations used for the myometrial standard were 0.5, 2, 5, 20 and 40μg/ml of protein, and for the recombinant HSP27 protein; dilutions from 128 - 1ng/ml. The lowest concentrations of the myometrial sample (0.5 and 2μg/ml) gave readings which were at background values. OD values for the recombinant HSP27 protein at concentrations between 4.0-64.0ng/ml equated closely with each other when adjusted on an absorbance per ng basis. The top concentration of 128ng/ml did not equate closely, giving an OD reading of 6.96 and falling outwith the linear part of the curve.

When myometrial OD readings were similarly equated to give an absorbance value per μg, the readings did not equate as closely. This observation was confirmed when TMB was used as the substrate. Both standards were incorporated into each ELISA but the recombinant protein standard was used to equate values for the ovarian cell lines. Using the recombinant protein also had the advantage of expressing values in ng of HSP27 rather than myometrial 'units'.

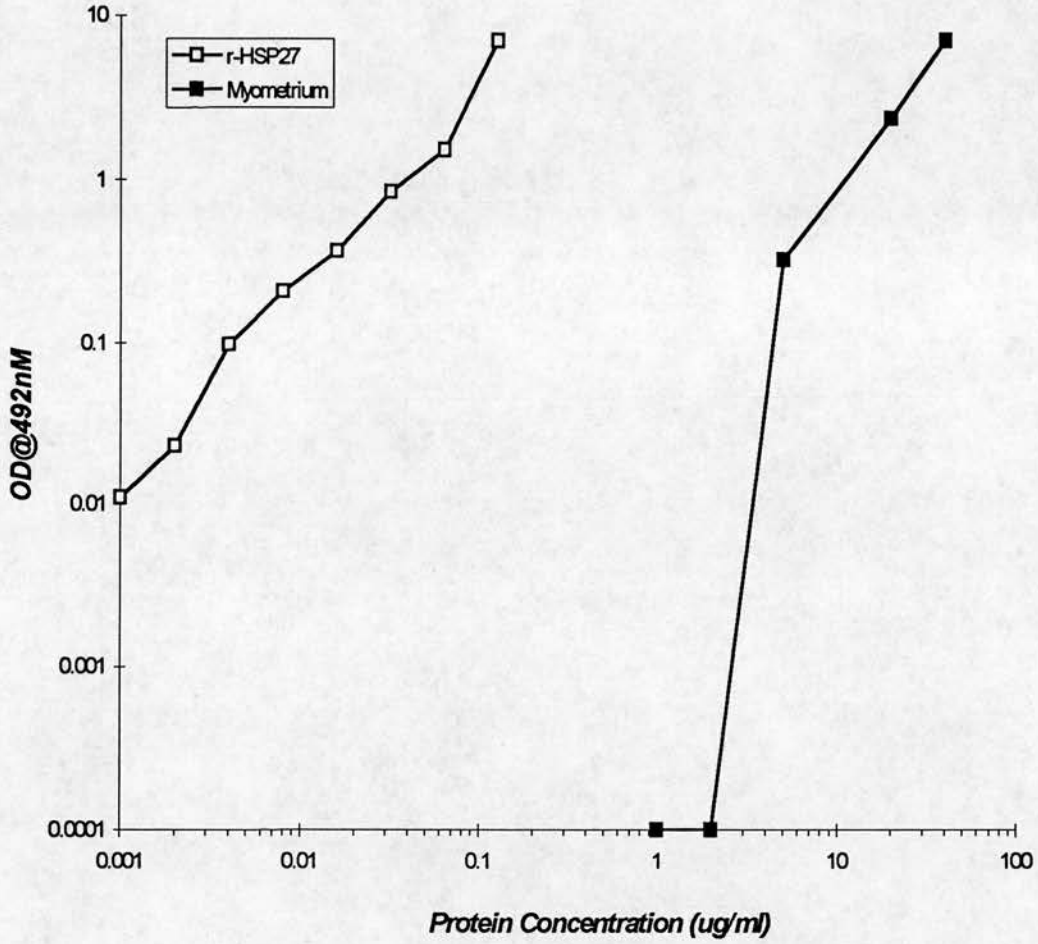


Figure 3.33

HSP27 expression measured by optical density at 492nM in titrations of myometrium and recombinant HSP27. Results shown are from one experiment.

c) Sample Dilution

A range of dilutions for each cell line cytosol was evaluated using the protein levels calculated for each sample. Results following substrate optimisation determined suitable protein ranges for ZR-75-1 cells. Figure 3.34 shows typical optical densities obtained for a range of dilutions of both control and oestrogen-treated PEO4 and PEO14 cells run in the ELISA using TMB as the peroxidase substrate. Also shown are typical values for the recombinant HSP27 protein, and control and oestrogen-treated ZR-75-1 breast cells. The graph shows that HSP27 expression in the cell lines appeared to dilute similarly to the recombinant protein, although values for the ZR-75-1 breast cancer cell line equated more closely than the ovarian cancer cell line readings when adjusted per μg of protein. The recombinant HSP27 was run in each assay at 50, 25 and 10ng/ml to provide a range of OD readings. Protein concentrations at 40, 20 and 10μg/ml were chosen for the ovarian and MDA-MB-231 cell lines, and 6.25, 5.0, 3.75, 2.5μg/ml for the ZR-75-1 cell lines which gave OD values within the range for the recombinant protein, and falling within the linear range of the titration curve. The internal myometrial standard was also incorporated in each assay at 20, 10 and 5μg/ml.

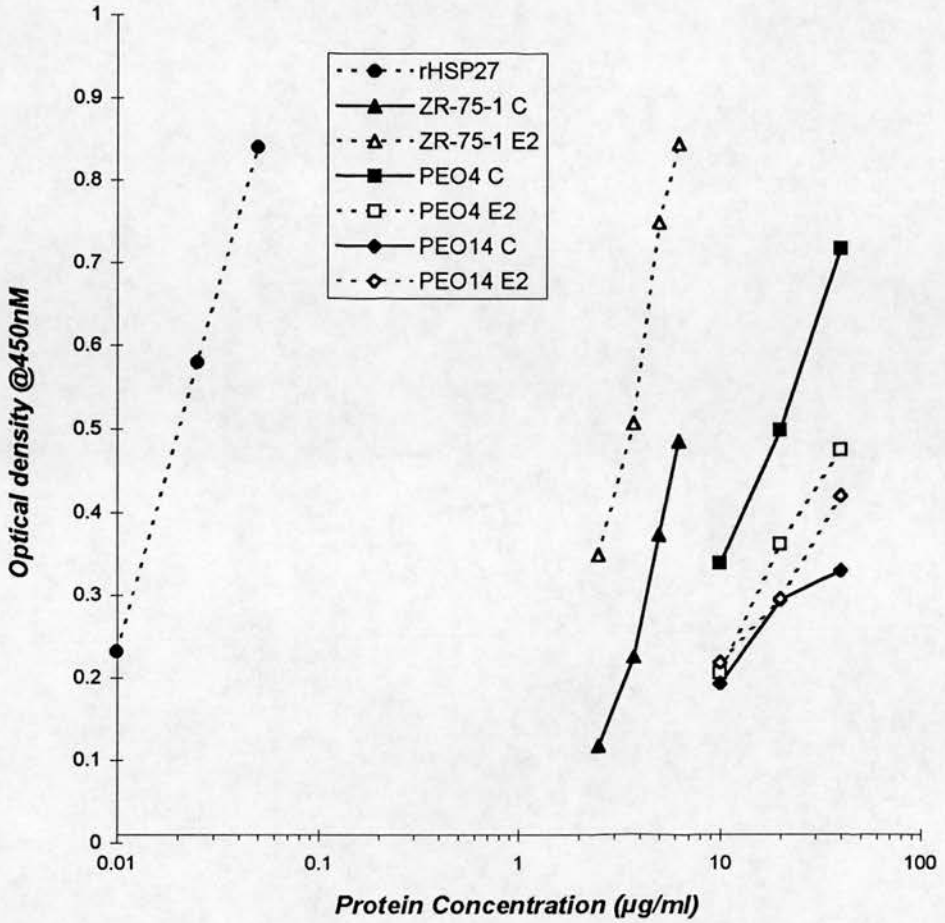


Figure 3.34

Detection of HSP27 expression in two ovarian cell lines and the breast cell line, ZR-75-1 at a range of protein concentrations. Cells were cultured in phenol red-free media containing 5% dcsFCS in the presence (open symbols, E_2) or absence (closed symbols, C) of 10^{-10}M 17 β-oestradiol for three days. Cytosols were then extracted and examined for HSP27 expression by ELISA. Results show typical optical densities read at 450nM from one experiment after subtraction of background values.

(ii) Effects of oestrogen on HSP27 expression *in vitro*

The effects of oestrogen on HSP27 expression were measured in several of the ovarian cell lines (PEO1, PEO4, PEO1^{CDDP} and PEO14), and the ZR-75-1 and MDA-MB-231 breast cell lines. Cells were grown in phenol red-free media supplemented with 5% dcsFCS either in the presence or absence of 10^{-10}M 17 β-oestradiol. After three days cells were removed and cytosols prepared as described in the Methods, section 2.28. Dilutions of samples were prepared as described in the previous section and analysed by ELISA incorporating TMB as the peroxidase substrate. Figure 3.35 illustrates the results of the analysis for the six cell lines which are summarised in Table 3.9.

At higher protein concentrations a plateau effect was sometimes observed with the dilution curve, therefore values for each cell line were calculated from the dilution giving an OD reading which fell on the linear part of the curve and within the OD range of the recombinant protein standard. This value was equated to an absorbance for 1μg of protein, and the concentration of HSP27 then calculated from the OD reading for 1ng of standard.

HSP27 expression levels above background were detected in both control and modulated groups of all the cell lines analysed. When considering either treatment group, the greatest level of expression was noted in the ER-positive ZR-75-1 breast cell line, this contrasting with the ovarian cell lines which expressed 3-8 fold lower HSP27 levels, and the ER-negative breast line, MDA-MB-231 which had a very low HSP27 content. In the ZR-75-1 line, the level of HSP27 expression was significantly

increased ($p<0.05$) in cells exposed to oestrogen, as compared to control cells grown in oestrogen-free conditions. Conversely, significant decreases in expression were noted in oestrogen-treated PEO4 and $PEO1^{CDDP}$ ovarian cell lines as compared to control cells. A slight increase in HSP27 content was observed in the PEO1 cell line in response to oestrogen but this proved to be non-significant. There was no change in expression in the ER-negative PEO14 or MDA-MB-231 breast cell line.

Table 3.9

Effects of oestrogen on HSP27 expression in ovarian and breast cancer cell lines. Results are expressed in ng and are equated against the mean absorbance for 1ng of recombinant HSP27 standard run in each assay. Each value represents the mean of nine separate experiments ± SEM. *p<0.05, **p<0.005 for the difference between control and treated cells, evaluated using a paired Students *t*-test.

Cell Line	HSP27 Concentration (ng/µg cytosolic protein)	
	Control	+ 10^{-10}M E_2
PEO1	0.516 ± 0.114	0.754 ± 0.197
PEO4	1.270 ± 0.112**	0.956 ± 0.095**
$PEO1^{CDDP}$	1.340 ± 0.151**	0.772 ± 0.102**
PEO14	0.558 ± 0.101	0.455 ± 0.061
ZR-75-1	4.470 ± 0.470*	6.830 ± 0.766*
MDA-MB-231	0.292 ± 0.022	0.254 ± 0.034

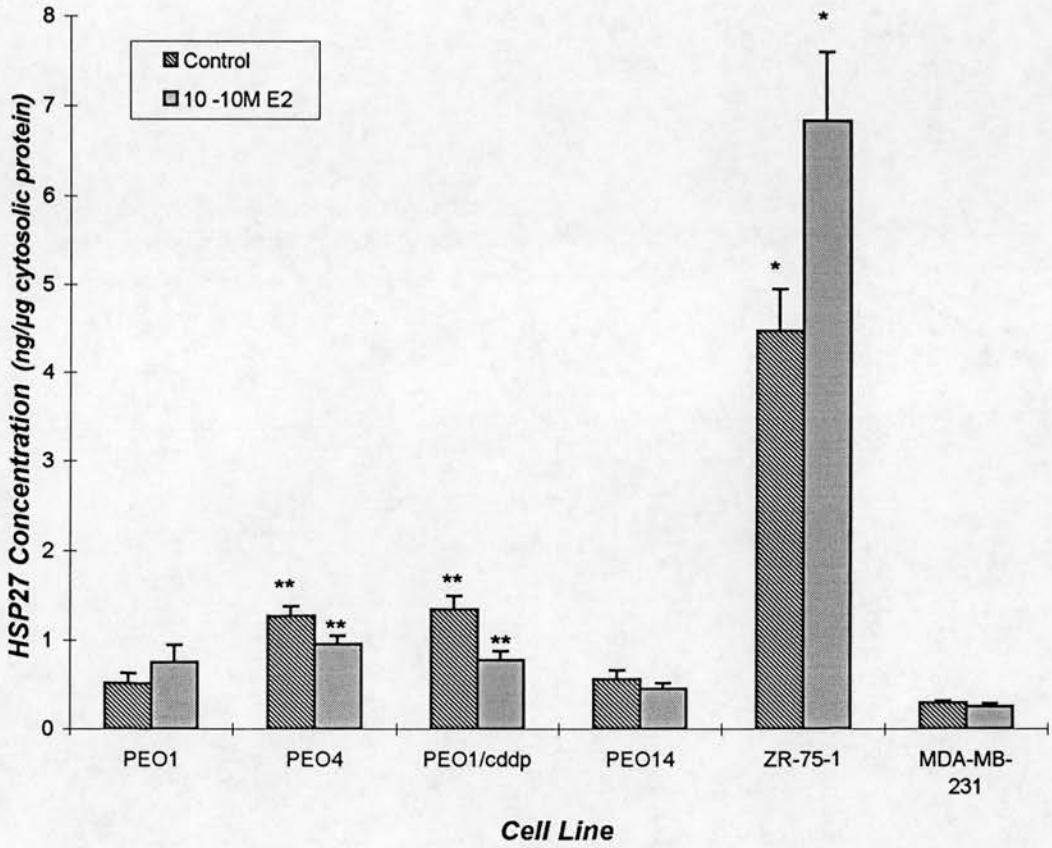


Figure 3.35

Effects of oestrogen on HSP27 expression in ovarian and breast cancer cell lines cultured in phenol red-free media supplemented with 5% dcsFCS. Striped bars represent cells which were grown in the absence of oestrogen and shaded bars represent those grown in the presence of 10^{-10}M 17 β-oestradiol (E_2). Results show the mean of nine separate observations ± SEM. *$p<0.05$ (ZR-75-1), **$p<0.005$ (PEO4 and $PEO1^{CDDP}$) for the difference between control and treated values, according to a paired Student *t*-test.

3.5.4 Effects of oestrogen on HSP27 expression in xenografts

The modulation of HSP27 expression was also investigated in the PEO4 xenograft model. Xenografts were established as described previously and treated tumours were exposed to a subcutaneously implanted 1.7mg 17 β-oestradiol slow-release pellet for 60 days. Control tumours were grown without additional oestrogen supplement. After this period, treated and untreated tumours were extracted and cytosols prepared as for the *in vitro* cell lines. TMB was used as peroxidase substrate in the ELISA to be consistent with *in vitro* experiments. Xenografts were initially titrated at a range of dilutions (4, 2, 1, 0.2µg/ml protein) to determine a suitable concentration range. Tumour samples appeared to dilute in parallel with titrations of the recombinant protein (Fig 3.36). Results were calculated using the criteria established in the *in vitro* experiments, i.e. dilutions which fitted the linear portion of the dilution curve and the recombinant protein standard curve.

Results for the oestrogen modulated and unmodulated xenografts are shown in Figure 3.37. HSP27 levels in the xenograft control group had a mean value of 5.7 ± 0.4ng and in the treated group, 5.1 ± 0.6ng, concentrations which were approximately five-fold greater than those observed in the *in vitro* PEO4 models (see Figure 3.35). However, unlike the situation *in vitro*, HSP27 concentrations in the PEO4 xenograft were not altered significantly in response to exogenous oestrogen treatment.

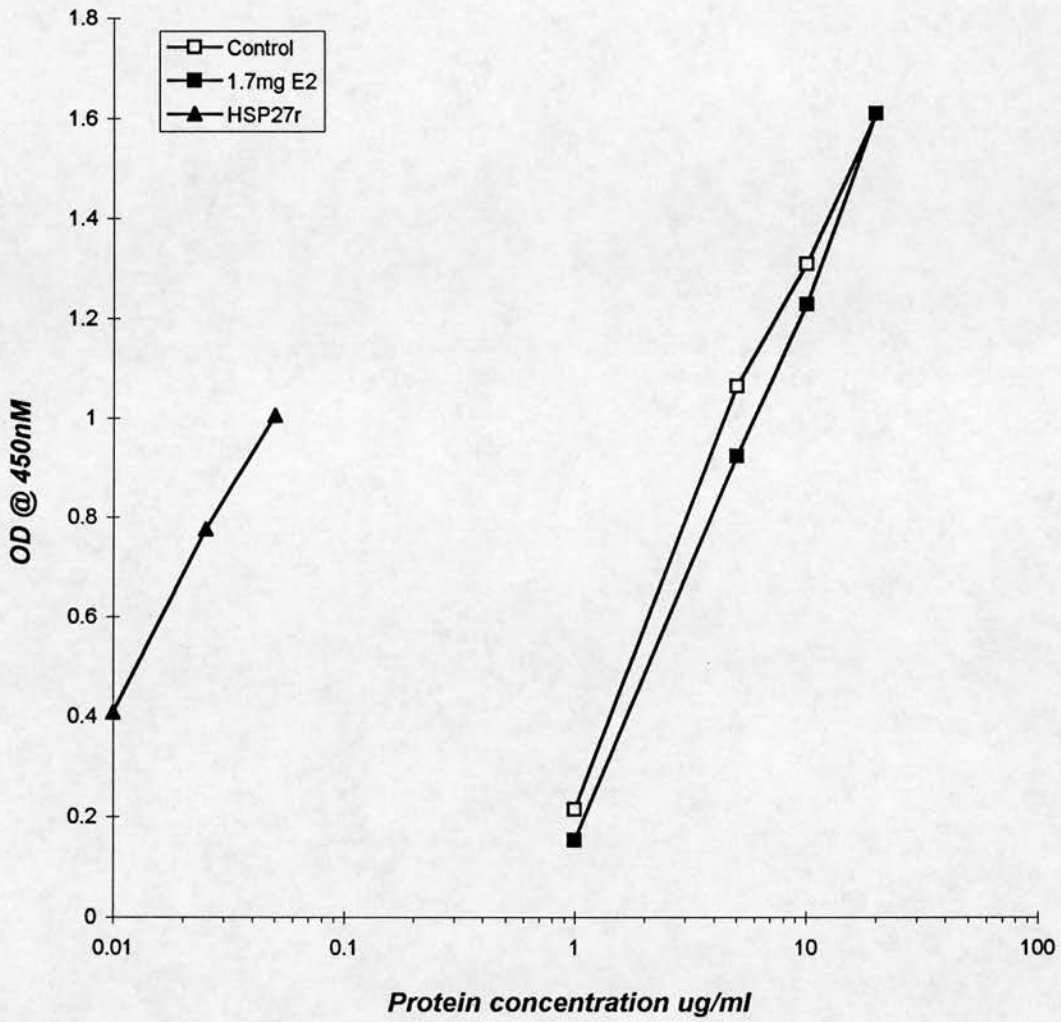


Figure 3.36

Typical dilution curve showing the absorbances at 450nM for different concentrations of untreated and oestrogen-treated PEO4 xenograft cytosols, run in the HSP27 ELISA. Also shown is a dilution curve of recombinant HSP27 protein.

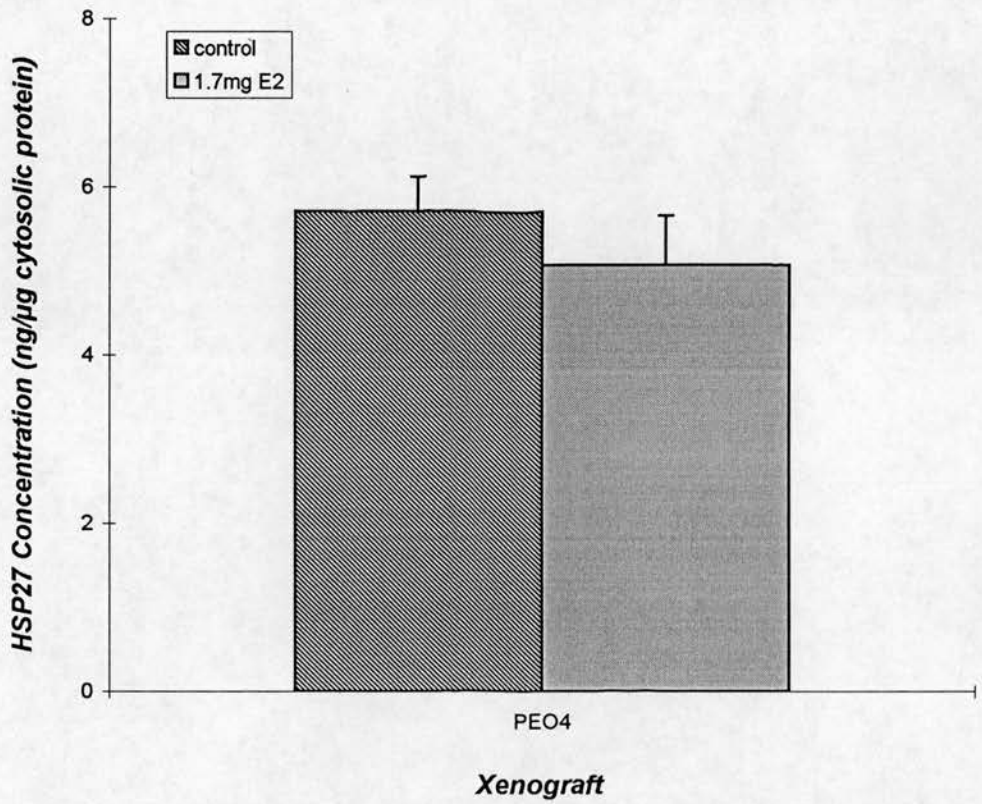


Figure 3.37

HSP27 levels in the PEO4 ovarian xenograft model grown in the absence or presence of a 60 day 17 β-oestradiol slow-release pellet. The striped bar depicts xenografts grown in the absence of oestrogen supplement, and the shaded bar represents oestrogen-treated xenografts. Results show the mean ± SEM of 9 separate observations and are expressed in ng HSP27/µg cytosolic protein, calculated from the ratio of the absorbances at 450nM of 1µg protein of sample cytosol with respect to 1 ng of recombinant HSP27.

3.6 Modulation of TGF-α and EGF expression in ovarian cancer cells

There is evidence from breast cancer studies to suggest that oestrogen may exert its growth stimulatory effects partly through the action of one or more growth factors, by altering their expression in target cells (Dickson *et al.*, 1986, Bates *et al.*, 1988, Huff *et al.*, 1988). In ovarian cancer, this may involve members of the EGF growth factor family, such as TGF-α and EGF acting through the EGF receptor. Previous studies have demonstrated the presence of EGF receptors in the PEO1, PEO4 and PEO14 ovarian cell lines which are also growth stimulated by TGF-α and EGF (Crew *et al.*, 1992a). If these cells are shown to secrete these factors, an autocrine or paracrine growth pathway may exist, which could be partly influenced by the action of oestrogen.

To determine whether oestrogen modulates the secretion of TGF-α and EGF, levels of these growth factors were measured in the conditioned media of PEO1, PEO4, PEO14 and $PEO1^{CDDP}$ ovarian cancer cell lines growing in the absence or presence of 10^{-10}M 17 β-oestradiol for three days. Cells were cultured in phenol red-free media without serum. Growth factors were detected in the conditioned media using liquid phase competitive radioimmunoassays (RIA) specific for human TGF-α and EGF. Both assays are based upon the competition of labelled ^{125}I-peptide and unlabelled standard or unknown, binding to a limited quantity of peptide-specific antibodies in each reaction.

3.6.1 TGF-α radioimmunoassay

Analysis of TGF-α was performed using TGF-α RIA kits obtained from Peninsula.

(i) Specificity

The Peninsula kit for TGF-α detection comes supplied with a rabbit anti-TGF-α (rat) antibody which the manufacturers claim shows a similar level of cross-reactivity (100%) for both human TGF-α and rat TGF-α, but shows no cross-reactivity with human EGF. To confirm the cross specificity of the rat TGF-α antibody and the suitability of the rat TGF-α standard in the assay, serial dilutions of human and rat TGF-α standards (10pg/ml-1280pg/ml) were prepared and incubated with fixed amounts of the TGF-α antibody, goat anti-rabbit IgG, ^{125}I-TGF-α (rat) and normal rabbit serum. In addition, a series of log dilutions of human EGF standard were also tested with the supplied TGF-α antibody.

Figure 3.38 shows standard curves for the rat and human TGF-α, and human EGF, generated from the concentration of each standard versus the % bound (B)/maximum binding (Bo) of labelled peptide. A 100% value demonstrates maximum binding of labelled peptide (^{125}I-TGF-α) and indicates a low level (limit of detection) of unlabelled standard. As the amount of unlabelled standard increases the %B/Bo value decreases indicating the greater competition for binding by the cold competitor. Similar curves were produced for both human and rat TGF-α standards denoting the same level of reactivity with the rat anti-TGF-α antibody. The curves generated also matched the standard curve profile provided with the kit. All dilutions of EGF produced a %B/Bo value for the ^{125}I-TGF-α of around 90-100% indicating very little

competition for binding from the EGF competitor, and thus little reactivity between the anti TGF-α antibody and EGF.

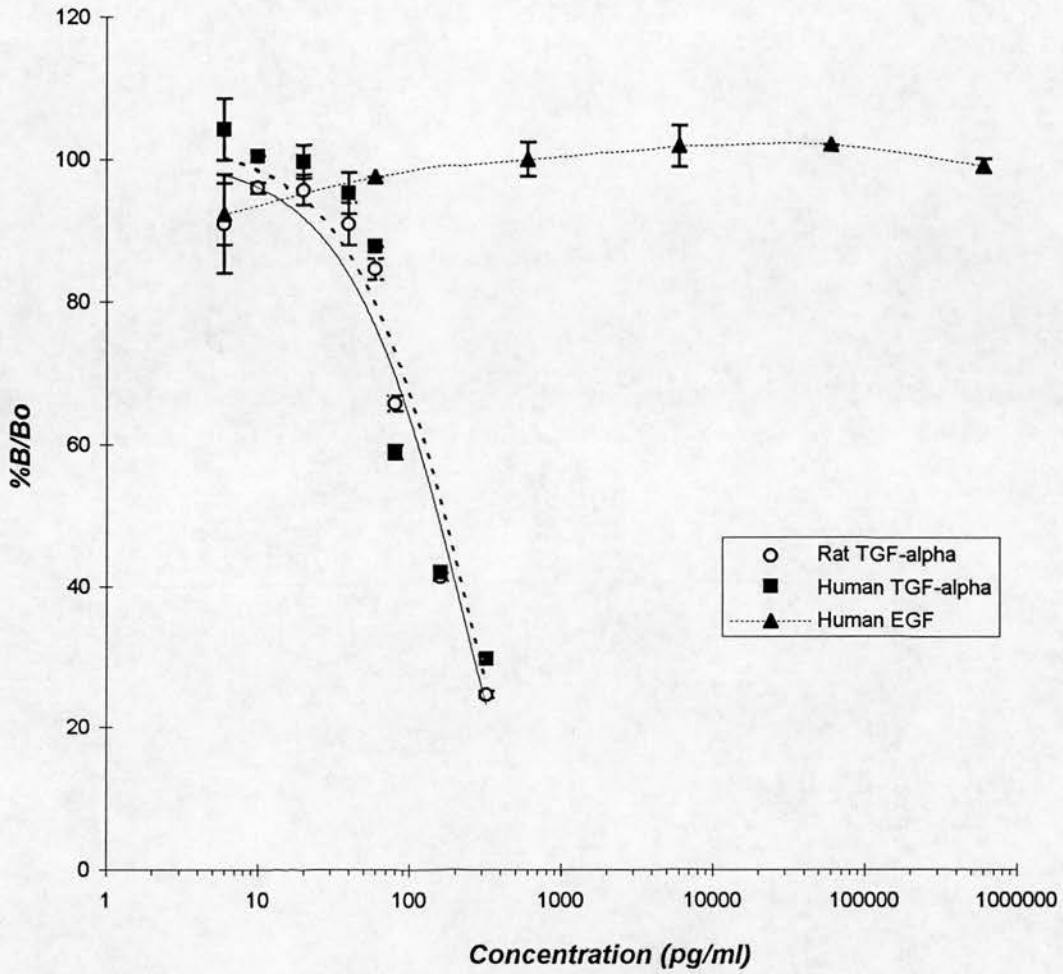


Figure 3.38

A standard curve calculated from the % bound (B) / maximum binding (Bo) of ^{125}TGF-α (rat) for different concentrations of unlabelled human (open symbols) or rat (closed symbols) TGF-α in a TGF-α radioimmunoassay. Readings were initially measured as counts per minute. The % bound value (B) was calculated by subtracting the non-specific background reading (binding of ^{125} TGF-α in the absence of primary antibody and unlabelled TGF-α) from each sample reading. Maximum binding, Bo, was calculated from binding of ^{125}TGF-α in the presence of both primary and secondary antibodies but without unlabelled TGF-α. Values shown are the mean of duplicate observations from one experiment, and bars represent SEM.

(ii) Sample preparation

a) Effect of concentrating samples

Because secreted levels of TGF-α are extremely low it has usually been necessary to concentrate and purify conditioned media samples by various methodologies in order to detect the peptide by conventional assays. In order to determine whether this would be necessary to detect secreted TGF-α in this study, conditioned media from cultured cells was concentrated by freeze-drying, and dialysed against PBS. Table 3.10 shows the results from a TGF-α RIA for unprocessed and processed (concentrated and dialysed) conditioned media samples from ZR-75-1 breast cells, as well as concentrated conditioned media from two ovarian cell lines, PEO1 and PEO4. Cell lines were grown in phenol red-free media in the absence of serum for three days, after which media was collected and processed.

Assays of unprocessed conditioned media from ZR-75-1 cells gave binding values of around 100%, which equated to a TGF-α concentration which was at the standard curve limit. When the cell conditioned media was concentrated 25-fold, a binding value of 88.8% was obtained, which fell within the range of the standard curve. After taking the concentration factor into account, this gave a mean final concentration of 4.1pg/ml. However, conditioned media from the ovarian cell lines which was concentrated 25-fold, had high %B/Bo values. TGF-α concentrations therefore could not be calculated for PEO1 or PEO4 which gave readings at the limit of the curve. These results suggested that media from the cell lines would have to be concentrated further in order to obtain a reading from the standard curve. Ideally, readings should

be taken from the plot where the relationship between TGF-α concentration and %B/Bo is linear. Conditioned media from the ovarian cell lines for subsequent analysis were concentrated 100-fold which gave readings falling within this range.

Table 3.10

% B/Bo and TGF-α concentrations of conditioned media from ZR-75-1 cells which was either unprocessed or concentrated 25 fold and dialysed. Also shown are values for processed media from the PEO1 and PEO4 ovarian carcinoma cell lines. Values shown are for duplicate measurements. Cells were cultured in phenol red-free media without 5% dcsFCS which were collected for analysis after three days.

Sample	%B/Bo	TGF-α Concentration (pg/ml)
Cell Lines		
ZR-75-1 unprocessed	100.7 ± 0.55	at curve limit
ZR-75-1 x 25	88.77 ± 0.63	4.06 ± 0.26
PEO1 x 25	95.50 ± 1.39	at curve limit
PEO4 x 25	95.76 ± 0.59	at curve limit

b) Effect of serum and protease inhibitors

Table 3.11 shows the % B/Bo values and concentrations of TGF-α like activity for conditioned media collected from breast and ovarian cell lines cultured in the presence or absence of 5% dcsFCS. Also shown are measurements for media alone which were prepared in the absence or presence of serum and protease inhibitors (0.2mM phenylmethanesulfonyl fluoride, 1mM EDTA, 0.5mg/L leupeptin and 0.7mg/ml pepstatin A).

Conditioned media from ZR-75-1 cells demonstrated TGF-α like activity both in the presence and absence of serum when concentrated 25-fold and incubated with protease inhibitors. Similarly, PEO1 and PEO4 cells grown in the presence of 5% dcsFCS demonstrated binding activity which was within the range of the standard curve, giving mean values of TGF-α-like activity of 3.95 and 4.32 pg/ml respectively. However, there was no detectable TGF-α like material in conditioned media obtained from PEO1 or PEO4 ovarian cells grown in the absence of serum.

Analysis with the non-cell conditioned media samples showed that RPMI or DMEM culture media alone did not exhibit any TGF-α like activity. But when 5% dcsFCS was present in the media, some TGF-α like activity was detected which was enhanced in the presence of protease inhibitors. Maximum binding was observed in the RPMI sample incubated just with protease inhibitors, suggesting that the inhibitors on their own did not exert any binding activity, but enhanced the competition for binding from any factors present in serum.

Because of the possibility of competition for binding in stripped serum, subsequent experiments in the cell lines determining oestrogen modulation of TGF-α were carried out in serum-free conditions. Conditioned media collected were incubated with protease inhibitors to prevent degradation of the growth factor, and concentrated 100-fold.

Table 3.11

(A) Shows the % B/Bo for conditioned media collected from ZR-75-1, PEO1 and PEO4 cell lines cultured with or without serum for three days. Media was concentrated 25-fold in the presence of protease inhibitors, and dialysed against PBS. Key: S (5% dcsFCS), P (protease inhibitors). Values shown are the mean of duplicate experiments ± SEM.

Sample	%B/Bo	TGF-α concentration (pg/ml)
(A) Cell Lines		
ZR-75-1+ P	88.77 ± 0.63	4.06 ± 0.26
ZR-75-1 + S + P	86.89 ±1.91	4.79 ± 0.75
PEO1 + S + P	89.02 ±1.50	3.95 ± 0.61
PEO1 + P	95.50 ± 1.39	at curve limit
PEO4 + S + P	88.11± 1.23	4.32 ± 0.49
PEO4 + P	95.76 ± 0.59	at curve limit
(B) Media samples		
RPMI only	99.96 ± 0.54	at curve limit
DMEM only	100.4 ± 0.4	at curve limit
RPMI + S	91.86 ± 0.36	3.67 ± 0.41
RPMI + S + P	85.10 ± 1.20	6.55 ± 0.25
RPMI + P	108.65 ± 2.95	at curve limit

3.6.2 Modulation of TGF-α secretion by 17 β-oestradiol

The level of TGF-α-like activity was examined in the conditioned media of four ovarian cell lines, PEO1, PEO1^{CDDP}, PEO4 and PEO14 which were cultured in the absence or presence of 10^{-10}M 17 β-oestradiol, and in the absence of serum for three days. Protease inhibitors were added to the conditioned media collected, which was

concentrated 100-fold. At this concentration, detectable levels of TGF-α were measured in conditioned media from the ovarian cell lines. Figure 3.39 shows typical %B/Bo values obtained for the TGF-α RIA, and confirms the presence of TGF-α-like activity in titrations of conditioned media from untreated and treated ZR-75-1 and PEO4 cells, which dilute in parallel with a typical standard curve of known concentrations of TGF-α.

Results of TGF-α RIA's for the untreated and treated ovarian and breast cell lines are given in Figure 3.40 and are summarised in Table 3.12. TGF-α-like activity was detected in the conditioned media from all the cells after 72 hours culture, but was at an extremely low level in conditioned media from the ER-negative PEO14 ovarian cell line. Higher levels of TGF-α-like material were secreted by the ER-positive ovarian cell lines, PEO1, PEO4 and $PEO1^{CDDP}$. Moreover, in two of these lines, PEO4 and $PEO1^{CDDP}$, secreted levels were significantly increased from cells which were exposed to 10^{-10}M 17 β-oestradiol as compared to cells cultured in oestrogen-free conditions. Levels of TGF-α-like activity also appeared to be increased in the PEO1 cell line (near significance, $p = 0.051$). In addition, TGF-α-like material was detected in the conditioned media of ER-positive ZR-75-1 cells, but there was no significant difference in the levels of secreted TGF-α from either untreated or oestrogen-treated cells. Cell counts of the above cell lines growing in serum-free conditions in 24 well plates for 72 hours indicated that there was no significant increase in cell number over this period (data not shown), thus suggesting that the increase in TGF-α in the

conditioned media of oestrogen-treated PEO4 and cells was due to increased secretion and not a greater number of cells producing the growth factor.

Table 3.12

Detection of TGF-α-like activity in the conditioned media of four ovarian and one breast cell line. Cells were cultured in serum-free RPMI (or DMEM; ZR-75-1) without phenol red in the absence or presence of 10^{-10}M 17 β-oestradiol (E_2) for 72 hours. Conditioned media were then collected, concentrated x100 and analysed using a RIA specific for TGF-α. Results are expressed as pg of TGF-α/ml of media. Values shown are the mean of three separate measurements ± SEM. *p= 0.051, **p<0.05

Cell Line	TGF-α concentration (pg/ml)	
	Control cells	+ 10^{-10}M E_2
PEO4	1.23 ± 0.18**	4.93 ± 1.23**
$PEO1^{CDDP}$	1.55 ± 0.12**	6.60 ± 1.09**
PEO1	0.87 ± 0.19*	1.60 ± 0.19*
PEO14	0.24 ± 0.15	0.23 ± 0.12
ZR-75-1	3.45 ± 0.67	3.00 ± 0.29

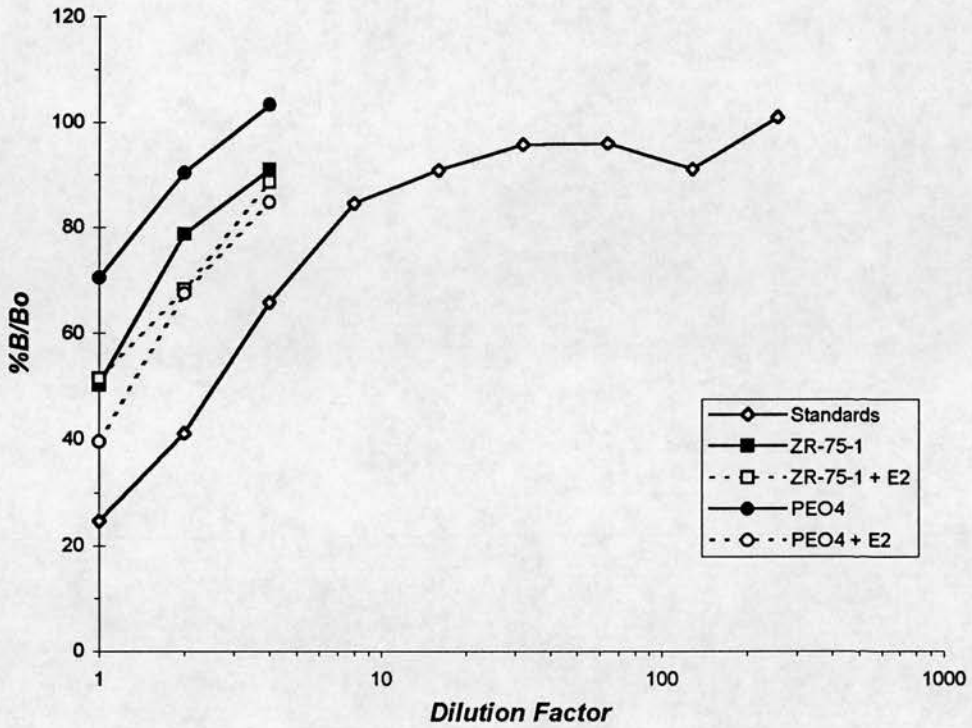


Figure 3.39

Typical displacement curves showing the presence of TGF-α like activity in serial dilutions of conditioned media from untreated and oestrogen-treated PEO4 and ZR-75-1 ovarian and breast cells, against a range of known TGF-α concentrations.

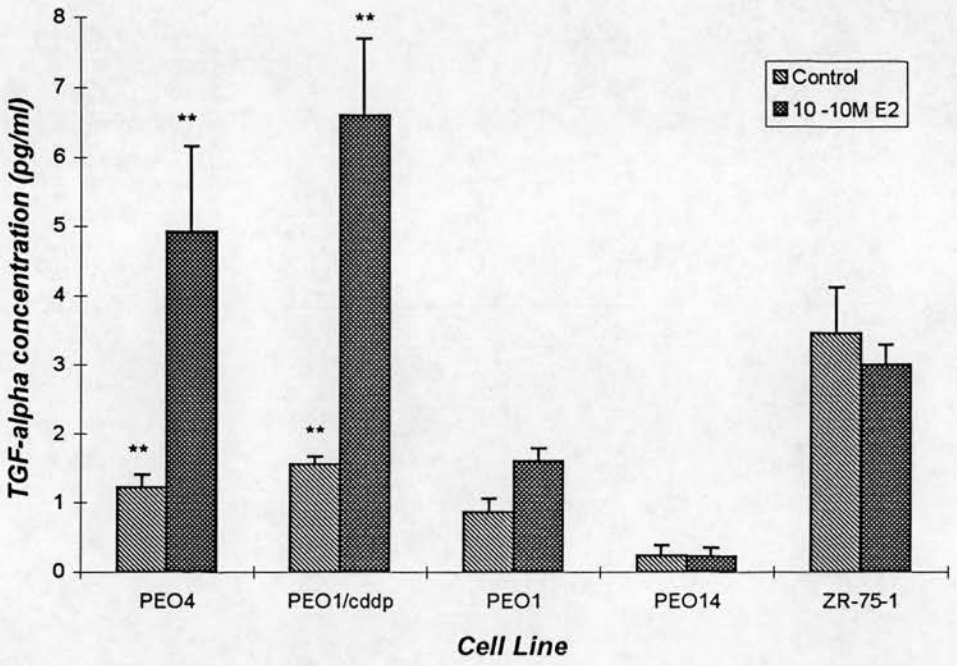


Figure 3.40

TGF-α-like activity in the conditioned media of four ovarian and one breast cell line, cultured in the absence or presence of 10^{-10}M 17 β-oestradiol for 72 hours. Conditioned media was then collected, concentrated and analysed using a TGF-α specific RIA. Lighter shaded bars represent untreated cells and darker shaded bars treated cells. Values shown represent the mean of three separate observations and error bars signify SEM. Statistical significance, **$p < 0.05$, *$p = 0.05$ evaluated by a Student's *t*-test.

3.6.3 EGF Radioimmunoassay

(i) Specificity

The radioimmunoassay for EGF was adapted from a method described by McDonald *et al.*, (1990), incorporating a sheep anti-human EGF antibody which does not cross react with murine EGF or human TGF-α. Initial experiments were set up to confirm this by incubating both a series of human recombinant EGF and TGF-α standards (0.2-200 ng/ml) with the anti-EGF antibody and ^{125}I labelled human EGF. The results shown in Figure 3.41, demonstrate that the EGF standards effectively compete out binding by labelled EGF in a dose dependent manner. However, as indicated by the maximum %B/Bo values, no competition for binding is exhibited by any of the TGF-α standards.

(ii) Sample Preparation

a) Effect of concentrating samples

Previous reports with this assay have shown that concentration of conditioned media was necessary in order to detect the low levels of EGF-like activity produced by a prostatic cancer line, DU145 (McDonald *et al.*, 1990). Therefore, initial RIA's were performed with cell line conditioned media from ZR-75-1, and three ovarian cell lines which had been cultured in the absence of serum Samples were incubated with protease inhibitors and concentrated 25-fold. Results indicated that no detectable EGF-like activity could be measured in these samples, implying further concentration might be necessary to detect the presence of the growth factor.

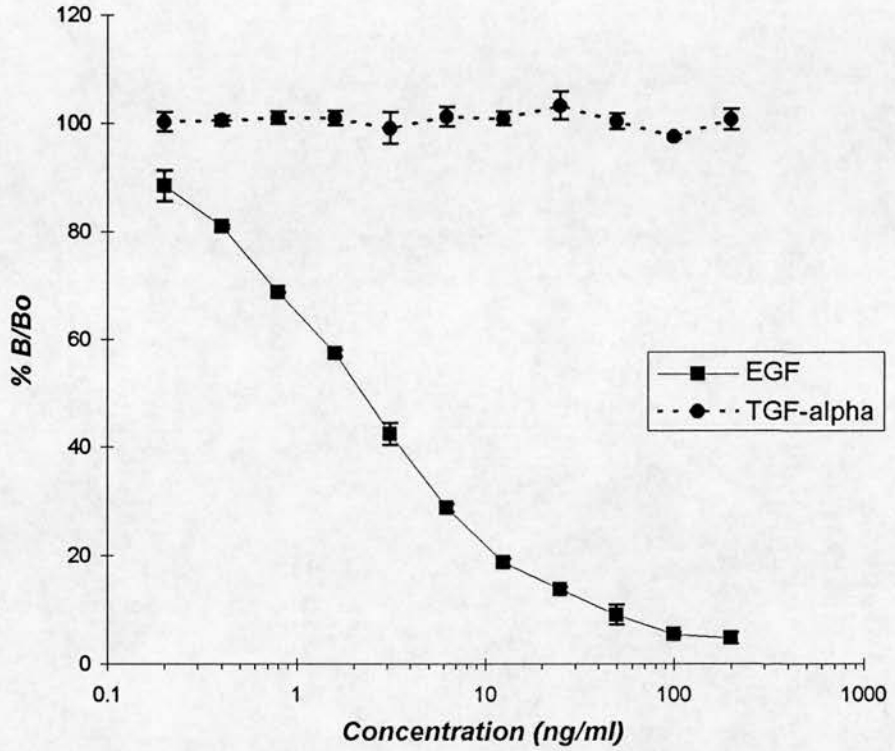


Figure 3.41

Graph showing specificity of the anti-human EGF antibody for human recombinant EGF, but no cross reactivity with human recombinant TGF-α. Standards were incubated with ^{125}I EGF and sheep anti-human EGF antibody, followed by incubation with donkey anti-sheep polyclonal IgG. Bound fractions were counted by gamma counter, and from this %B/Bo values calculated as described previously.

b) Effect of serum and protease inhibitors

RPMI media samples containing either 5% dcsFCS or protease inhibitors, or both were run in the EGF assay to determine whether these factors elicited any binding activity. In addition, conditioned media from ZR-75-1, PEO1, PEO4 and PEO14 cells grown in the presence of 5% dcsFCS and protease inhibitors were also run. Samples were concentrated 25-fold and dialysed against PBS. No detectable EGF-like activity was seen in RPMI alone or with the addition of protease inhibitors. In contrast to the TGF-α RIA, the presence of 5% dcsFCS in media did not elicit any competition for binding in the EGF RIA, either alone or with protease inhibitors. At 25-fold concentration, conditioned media from any of the cell lines assayed did not contain detectable levels of EGF activity when cells were grown in the presence of 5% dcsFCS and protease inhibitors.

3.6.4 Modulation of EGF secretion by 17 β-oestradiol

The same conditioned media samples which demonstrated detectable TGF-α levels, at 100-fold concentration, were also analysed for EGF activity using the EGF specific RIA. However, no EGF-like material could be detected in either untreated or oestrogen-treated samples using the assay which is sensitive in the range 0.2 - 200 ng/ml.

To further test whether the cell lines produced any detectable EGF-like material, two EGF standards used to create the RIA standard curve were diluted in the control conditioned media from PEO4 and PEO14. However, as can been seen in Figure 3.42, no displacement of the standard curve was seen, suggesting that the cell lines were secreting little or no EGF-like activity.

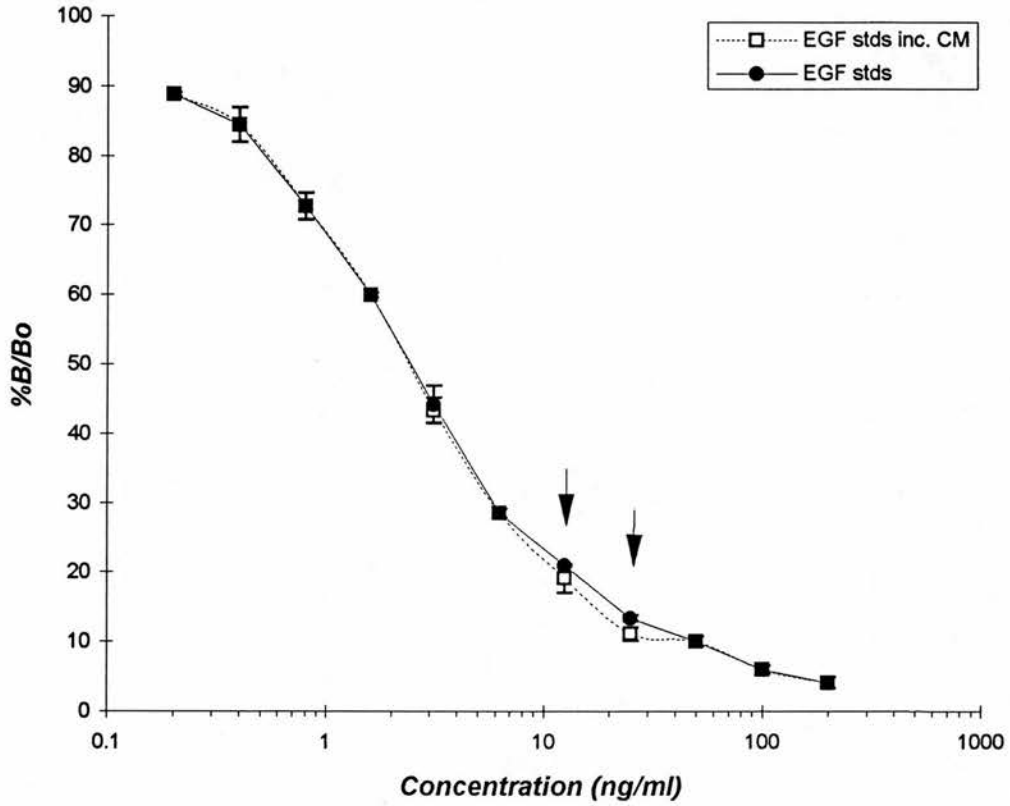


Figure 3.42

EGF standard curve containing a range of concentrations of human EGF. Arrows indicate where standards were spiked with conditioned media from PEO4 and PEO14 cells. Values shown are the mean of duplicate readings ± SEM.

3.7 Analysis of IGF-I Receptor regulation by oestrogen

Previous reports have implicated the insulin-like growth factors (IGF's) in the control of breast cancer growth through autocrine and paracrine growth pathways, (Yee *et al.*, 1988, Osborne *et al.*, 1989), and more recent evidence implies that they may also be involved in the growth regulation of ovarian carcinoma. IGF-I and its receptor is expressed by several ovarian carcinoma cell lines (Yee *et al.*, 1991a), including OVCAR-3 which is also growth stimulated by IGF-I (Yee *et al.*, 1991a, Resnicoff *et al.*, 1993). The receptor has also been demonstrated in ovarian tumours (Foekens *et al.*, 1990b). Recent data by Krywicki *et al.*, (1993) has shown that E_2 can downregulate the expression of IGF binding proteins in the PEO4 ovarian cell line, and suggested that this may make the cells more responsive to IGF-I. There is however still a paucity of data on the regulation of the IGF family by oestrogen in ovarian cancer and it was therefore of interest to examine whether the IGF-I receptor is modulated by oestrogen in ovarian cell lines. The PEO1, PEO4 and PEO14 cell lines all express mRNA for IGF-I and its receptor, and the presence of receptors has been confirmed by immunohistochemistry, Bartlett *et al.*, (1995).

Cell lines were grown in phenol red-free media supplemented with 5% dcsFCS and exposed to 10^{-10} M E_2 for six days, following which the IGF-I receptor levels were measured by flow cytometry. Figure 3.43 shows the percentage of cells staining for IGF-I receptor in the ER-positive PEO1, PEO4 ovarian lines and the ER-negative MDA-MB-231 breast cell lines in control and oestrogen-treated groups. Greater than

90% of cells in the ovarian cell lines stained positively for presence of the receptor. There was no significant difference between the percentage staining seen in the oestrogen-treated PEO1 and PEO4 cells and that observed in control cells. A much lower level of staining was seen in the ER-negative breast cell line with only approximately 7% of cells in the control group showing positive staining for the presence of the receptor. A smaller percentage of oestrogen-treated MDA-MB-231 cells had detectable IGF-I receptor (approximately 2%), although again this was non-significant.

Figure 3.44 shows the intensity of staining for IGF-I receptor measured in the cell lines calculated as a ratio of the mean fluorescence for each sample compared to the background. Both the ovarian ER-positive cell lines demonstrated a six to seven fold higher staining intensity than that seen in the ER-negative breast cell line. Intensity levels seen in oestrogen-treated PEO4 cells were significantly greater than PEO4 cells cultured without oestrogen. The mean staining intensity was also greater in oestrogen-treated PEO1 cells although this difference did not achieve significance. There was no difference between staining intensities seen in control and oestrogen-treated MDA-MB-231 cells.

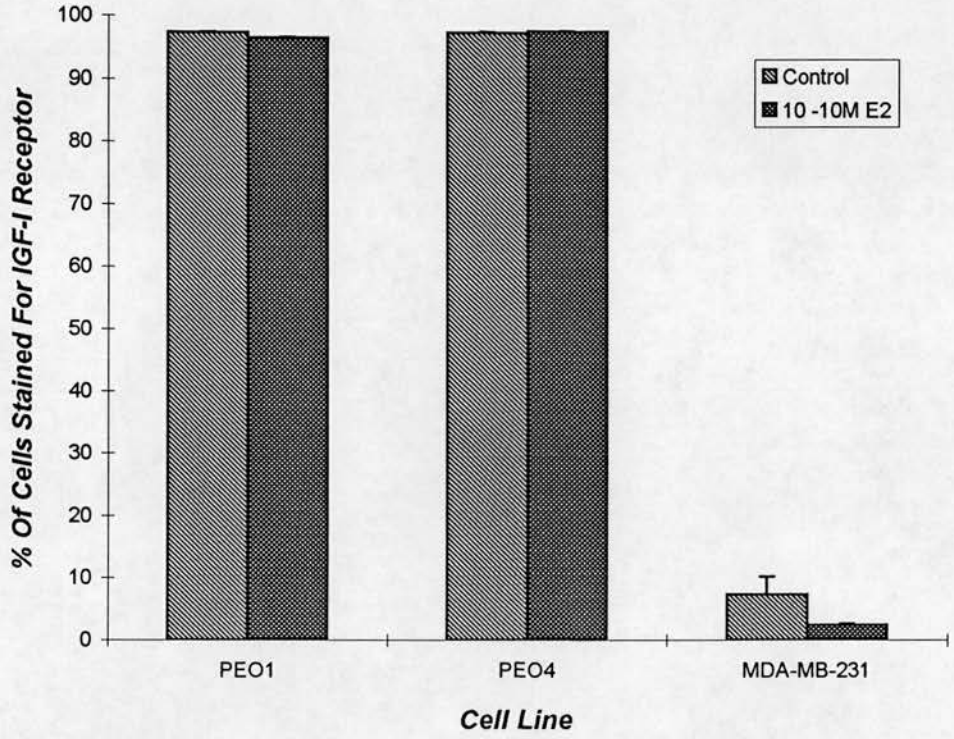


Figure 3.43

IGF-I receptor expression in two ovarian and one breast cell line cultured in phenol red free media supplemented with 5% dcsFCS. Darker shaded bars show cells which were grown in the presence of 10^{-10}M 17 β-oestradiol for six days and lighter shaded bars show cells which were untreated. Values are given for the percentage of cells staining positively for presence of IGF-I receptor and represent the mean of duplicate observations ± S.E.M.

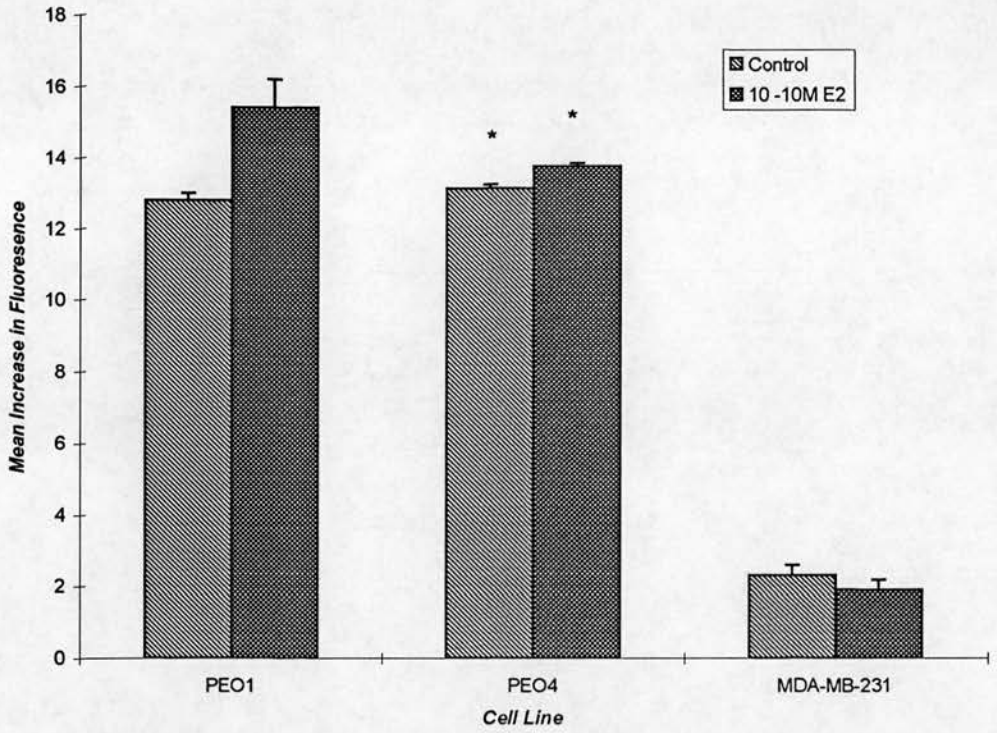


Figure 3.44

IGF-I receptor expression in two ovarian and one breast cell line cultured in phenol red-free media supplemented with 5% dcsFCS. Darker shaded bars show cells which were grown in the presence of 10^{-10}M 17 β-oestradiol for six days and lighter bars show cells which were untreated. Values are given for the intensity of positive staining for the IGF-I receptor and represent the mean of duplicate observations ± S.E.M.*$p<0.05$ as measured by a student's *t*-test.

4. DISCUSSION

4.1 Growth modulation by oestrogen

There has been increased interest in recent years in the potential role of hormones in ovarian cancer. The majority of human ovarian adenocarcinomas are oestrogen receptor (ER)-positive (reviewed in Slotman and Rao, 1988) and there have been reports of some responses with hormonal therapy, such as the anti-oestrogen, tamoxifen (Myers *et al.*, 1981, Hatch *et al.*, 1991), which has been successful for a number of years in the treatment of breast cancer. It is likely therefore that a subset of ovarian carcinomas are oestrogen-regulated but their characteristics are undefined. Knowledge of the role of oestrogens in breast carcinogenesis and tumour progression has been greatly enhanced by use of a number of oestrogen-sensitive breast cancer cell lines, such as the MCF-7 and ZR-75-1 lines (Soule *et al.*, 1973, Engel *et al.*, 1978). However, a lack of similar models for ovarian cancer has meant there is less insight into hormonal influences in this disease. Despite several ovarian cell lines being derived and established in recent years (Fogh *et al.* 1977, Buick *et al.*, 1985, Sinna *et al.*, 1979) there have been few studies describing hormonal sensitivity. In part this is because little attention has been paid to the steroid receptor content of these lines, and if assayed for the presence of ER, the majority of the cell lines have proven to be receptor-negative (Woods *et al.*, 1979, Bénard *et al.*, 1985). Thus, there has been a lack of clinically relevant experimental models.

This study has incorporated a number of ovarian carcinoma cell lines with varying levels of ER, amongst them the first recorded cell lines (PEO1, PEO4 and PEO6) with moderate/high levels of ER of approximately 100 fmol/mg protein, as detected

previously by the dextran-coated charcoal ligand binding assay (Langdon *et al.*, 1988). Evidence from breast cancer studies suggests that higher ER values (greater than 100 fmol/mg protein) are associated with a greater response rate to endocrine therapy (McGuire, 1978), whereas the response rate is much lower with decreased levels of ER. Thus, these ovarian cell line models may be more representative of a clinically responsive tumour phenotype than those previously described by other groups. The other cell lines studied have lower ER values; PEA1 and PEA2 have an ER content of 23 and 12 fmol/mg respectively, whereas the remaining cell lines, PEO14, PEO16 and PEO23 are ER-negative. Cell lines were deemed ER-negative if their receptor levels were less than 5 fmol/mg protein, the ER assay detection limit.

In addition, a further cell line, $PEO1^{CDDP}$ was also investigated. This cell line, which has not been previously described in the literature, is a cisplatin-resistant subline, derived by continuous culture of PEO1 cells in increasing concentrations of cisplatin for six months and is approximately 5-fold more resistant to cisplatin than the parent line, PEO1 (Langdon, personal communication). $PEO1^{CDDP}$ therefore provided a useful model to compare with the PEO1 and PEO4 cell lines which were derived from the same patient before and after the onset of clinical resistance to cisplatin combination therapy. These two lines have LD_{50} values of 0.064μM and 0.2μM respectively (Wolf *et al.*, 1987).

The series of ovarian cell lines described in this thesis are amongst the few reported with positive ER levels (Langdon *et al.*, 1988, 1990). Two other established ER-positive ovarian cell lines have been previously described. The NIH:OVCAR-3

cell line, established from the malignant ascites of a patient with a poorly differentiated ovarian adenocarcinoma, was the first ovarian cell line reported to contain steroid receptors, expressing both ER and AR (androgen receptors) at 28 and 30 fmol/mg protein respectively, (Hamilton *et al.*, 1983). The lower limit of ER values in breast tumours which may be hormonally sensitive is usually taken to be 20-30 fmol/mg protein, thus, the receptor level in the NIH:OVCAR-3 line just borders on the clinically significant. The other ER-positive ovarian cell line, BG-1, was characterised by Geisinger *et al.* (1989), and has a slightly lower ER content of 23 fmol/mg protein. A recent study by Hua *et al.*, (1995) has shown that the SKOV3 ovarian carcinoma cell line also has detectable levels of ER as measured by Western blotting.

The PEO series of cell lines, therefore provides a good experimental system with which to assess the effects of oestrogen, representing primary ovarian tumours with both lower and higher levels of ER. Two breast cancer cell lines were also included in this study; ZR-75-1, an ER-positive breast cell line, and MDA-MB-231, an ER-negative cell line, so that comparisons could be made between observations with the ovarian cancer cell lines and already established oestrogenic effects in breast cancer. The hormonal growth sensitivity of the PEO1, PEO4 and PEO14 had previously been characterised by Langdon *et al.*, (1990) but effects of oestrogen on the other cell lines had not been described before. To determine the effects of oestrogen on growth, all experiments were conducted in oestrogen-depleted conditions. Previous studies have shown that there are substantial levels of oestrogens in commercial foetal calf sera (FCS) (up to 10nM concentration) (Esber *et al.*, 1973),

as well as sulphate conjugates, and other steroids. These can be removed by using dextran-charcoal treated FCS (Stanley *et al.*,1977, Darbre *et al.*,1983). In addition, the pH indicator phenol red, normally present at high concentrations in culture media, is a weak oestrogen which can bind to the ER with an affinity 0.001% that of oestradiol (Berthois *et al.*, 1986). All experiments therefore, were carried out using phenol red-free media supplemented with double charcoal-stripped sera.

Under these conditions, growth of PEO4 and PEO14 cells has been shown to be reduced as compared to cells growing in normal culture media (Crew, personal communication). The absence or presence of phenol red indicator does not seem to make any difference to growth rates when cells are grown in charcoal-stripped serum (Langdon *et al.* 1990), thus this effect appears to be due to growth promoting agents being removed from the sera. One of these components is likely to be oestrogen as this can increase the growth rate in PEO4 cells when added back to the culture media (see next paragraph), however as the same effect is not observed with PEO14 cells, it would appear that other growth promoting agents such as other steroids or growth factors are removed as well.

Growth curves for the ER-positive PEO4 and the ER-negative PEO14 in the presence and absence of serum were initially reproduced to verify previous reports of their oestrogen sensitivity/insensitivity, and compared with the response of an ER-positive and ER-negative breast cancer cell line, ZR-75-1 and MDA-MB-231 to oestrogen. Both concentrations of 17 β-oestradiol used (10^{-10}M and 10^{-8}M) produced significant increases in growth over six days in the ER-positive PEO4 cell line as

compared to control cells, which was comparable to previous findings. There was also a 70% increase in growth of PEO4 cells in the absence of oestrogen over the time course. This might possibly be due to residual oestrogens in the culture media through insufficient stripping of sera, or inadequate washing with PBS. However as tamoxifen has previously been shown not to inhibit this growth (Langdon *et al.*, 1990), it appears that this residual growth activity is not due to oestrogenic stimulation. This suggests that the growth of PEO4 cells may be influenced by other mitogenic factors, additional to oestrogen, which are present in the culture medium.

The magnitude of growth response observed with oestrogen-treated PEO4 cells was comparable with that of the ER-positive ZR-75-1 breast cancer cell line which was significantly stimulated by the two concentrations of 17 β-oestradiol, consistent with previous observations that this is an oestrogen-sensitive cell line (Darbre *et al.*, 1983). However, very little growth was observed with control ZR-75-1 cells over the six days, a finding which is in keeping with reports that ZR-75-1 cell line is an oestrogen-dependent line (Glover *et al.*, 1988). This group found that there was some growth of ZR-75-1 cells in short term culture in oestrogen-free conditions (phenol-red free media plus 5% stripped FCS), but basal growth was reduced to zero after prolonged culture (2 weeks or more). If oestrogen was added back to the culture, basal cell growth returned, suggesting that growth of ZR-75-1 cells is dependent upon oestrogenic stimulation. They suggested that any residual growth which was seen in short term culture without the presence of oestrogens could have been due to a steroid "memory effect". No significant increase in growth was noted

with the control ZR-75-1 cells described in this thesis, suggesting that all oestrogens had been removed from the media.

Consistent with its lack of ER, there was no effect of oestrogen on PEO14 cell numbers. However, both control and treated cells increased approximately three-fold in number over the six days, a growth rate greater than that observed with control PEO4 cells which in normal culture conditions (phenol red, 10% FCS) have a shorter doubling time than PEO14 (Langdon *et al.*, 1988), suggesting that growth promoting factors for PEO14 are still present in dcsFCS. This response was similar to that seen with the ER-negative MDA-MB-231 breast cancer cell line which showed a four-fold increase in number over the six days.

The effect of varying concentrations of oestrogen on cell numbers was examined in eight of the ovarian cell lines to determine the range of hormonal sensitivity. Significant increases in growth were observed in the PEO1, PEO4 and PEO6 cell lines between the oestrogen concentration range 10^{-12} and 10^{-6}M, maximal stimulation being obtained in the range 10^{-10} to 10^{-8}M. This is in agreement with Nash *et al.*, (1989a) who compared the oestrogen sensitivities of NIH:OVCAR-3 and PEO4 lines, reporting the PEO4 line to be growth stimulated by concentrations of oestrogen between 10^{-12} and 10^{-8}M, with maximal effects at 10^{-10}M.

The increase in growth stimulation gradually fell between 10^{-8}M and 10^{-6}M, possibly caused by a down-regulation of ER. This is similar to oestrogen-sensitivity profiles obtained with ER-positive breast cancer cell lines (Lippman *et al.*, 1976, Darbre *et al.*, 1983). Sensitivities at concentrations lower than 10^{-12}M were not examined

except in the PEO1^{CDDP} line (see below), but Langdon *et al.*, (1993) has shown that the PEO4 cell line is not significantly growth stimulated by a dose of 10^{-13}M oestrogen. This sensitivity to oestrogen is consistent with the presence of moderate-high levels of ER in these lines (around 100 fmol/mg protein). Conversely, no increase in cell numbers was seen in response to oestrogen treatment in the PEA1 or PEA2 cell lines, which although being ER-positive, contain much lower levels of ER; 18 and 8 fmol/mg protein respectively when measured by enzyme immunoassay (see next section of discussion). This observation is compatible with reports in breast cancer which suggest that ER values greater than 20-30 fmol/mg protein delineate tumours which are likely to be hormonally sensitive (Jensen *et al.*, 1976). As might be expected with a lack of ER, there was no growth stimulation in either the ER-negative PEO16 or PEO23 cell lines.

There are only a few reports of oestrogen effects on the growth characteristics of other ovarian carcinoma cell lines. The BR ovarian carcinoma cell line in which the ER status is unknown is stimulated by several concentrations of oestrogen (Wimalasena *et al.*, 1993). Of the previously reported ER-positive lines, only the NIH:OVCAR3 and BG-1 lines show an increase in growth in response to oestrogen. Chien *et al.*, (1994) showed that the NIH:OVCAR-3 cell line was growth stimulated by oestrogen in contrast to an earlier finding by Nash *et al.*, (1989a), and effects of oestrogen on the proliferation of ER-positive BG-1 ovarian cells were noted by Galtier-Dereure *et al.*, (1992), who demonstrated that the growth of BG-1 cells was significantly increased after exposure to 17 β-oestradiol for 5 days. However, despite having detectable levels of ER, oestrogen has no effect on the growth of the SKOV3

line, (Hua *et al.*, 1995). Therefore, cell lines with low levels of ER around 20-30 fmol/mg protein such as BG-1 and NIH:OVCAR-3 may be oestrogen responsive. A report by Lazo *et al.*, (1984) showed that in clonogenic assays of cells taken directly from clinical ovarian samples, 2 out of 4 samples with an ER value of greater than 30 fmol/mg protein responded to the anti-oestrogen tamoxifen, but none of the samples with levels lower than 30 fmol/mg protein showed a response, again suggesting that cells with moderate to high levels of ER are more likely to be oestrogen sensitive.

An interesting observation was noted when the platinum-resistant subline $PEO1^{CDDP}$ was exposed to oestrogen. Although this line contains ER levels similar to the parental line PEO1, and these are down-regulated by 10^{-10}M 17 β-oestradiol to a similar degree (see section 4.2), concentrations of oestrogen which were stimulatory to the parent line caused a decrease in $PEO1^{CDDP}$ cell numbers. The magnitude of this inhibition gradually decreased at lower concentrations, until cell number was comparable with the control at a concentration of 10^{-16}M. The inhibitory effects of oestrogen on $PEO1^{CDDP}$ cells is not simply a cytostatic effect. Cell numbers increase after plating in both control and treated groups, but the rate of growth in treated cells appears to be less than those not exposed to oestrogen. After three days culture there is evidence of a growth inhibition in a dose dependent manner and following a further three days of oestrogen-exposure this is even more pronounced. Oestrogen is possibly causing a down-regulation of growth stimulatory mechanisms, or an up-regulation of growth inhibitory mechanisms.

This finding is certainly not consistent with the positive growth effects observed in the other cell lines with high levels of ER. The concentrations of oestrogen responsible for the inhibition were also not at levels which have previously been reported as being toxic to cells. In all the other cell lines, except PEO1, cytotoxic and cytostatic effects were only apparent with the highest concentration, 10^{-5}M, and also at a concentration of 10^{-6}M in PEO16. Although a concentration of 10^{-5}M produced a growth stimulation in PEO1, cytotoxic effects in PEO1 have been seen with a concentration of 10^{-4}M (Crew, personal communication). This is similar to observations by Reddel and Sutherland, (1987) who reported a 50% inhibition in the ER-positive MCF-7 breast cell line with 5 x 10^{-6}M 17 β-oestradiol, and suggested that high concentrations of oestrogens caused specific changes in cell cycle parameters. There appeared to be differences between different breast lines in that the ER-positive T47-D breast cancer cell line showed less sensitivity to inhibition by high dose oestrogen. However, they also noted that these observations were not limited to ER-positive lines in that an inhibitory effect was also noticed in the ER-negative cell line, MDA-MB-330 although the level of sensitivity was reduced. Likewise, there were no specific differences in cytotoxicity between ER-positive and ER-negative ovarian cancer cell lines in the present study, so it would seem that these effects are independent of the ER.

A report by Vickers *et al.*, (1988) observed that generation of drug resistance in human MCF-7 breast cancer cells by increasing concentrations of a drug such as adriamycin can lead to a loss of mitogenic response to oestrogen. This is associated with a loss of ER and other estrogen responses such as PR induction. In contrast, ER

expression is high in the $PEO1^{CDDP}$ cell line, but it may be possible that these receptors are dysfunctional, and the normal positive growth regulatory pathways are abrogated in some manner. As discussed later, PR levels are not induced in this line but other oestrogen modulated proteins such as HSP27 and TGF-α are. In addition, ER levels are downregulated, suggesting that the receptors are functional in $PEO1^{CDDP}$ cells.

Inhibitory effects of E_2 on growth have been observed in other ER-positive cell lines, including an MCF-7 variant (Bronzert *et al.*, 1984) and growth inhibition by oestrogen, at levels stimulatory to ER-positive breast cancer cell lines, has also been noted in human mammary epithelial cells, and ER-negative MDA-MB-231 breast cancer cells which have been transfected with a recombinant ER (Zajchowski *et al.*, 1993, Jiang and Jordan, 1992). Both these groups reported an increase of cells in the G_0/G_1 phase of the cell cycle, and a decrease in the S-phase cell population after E_2 treatment, increasing the cell doubling time. This could be noted as early as 12 hours in the transfected mammary line B5-ER (Zajchowski *et al.*,1993) and was observed after six days of treatment with 10^{-8}M E_2 in the transfected MDA-MB-231 cell line (Jiang and Jordan, 1992). The growth inhibition was blocked by the anti-oestrogen ICI 164,384 in both the transfected human mammary line, B-E23 (Zajchowski *et al.*, 1993), and the ER-transfected MDA-MB-231 (Jiang and Jordan, 1992). It was proposed that this growth effect may be attributable to the intolerance of ER overexpression in constitutively ER-negative cells. The inhibitory effects seen in $PEO1^{CDDP}$ are unlikely to be due to high ER expression as the parental PEO1 cells express similar levels of the protein. However, Jiang and Jordan (1992), suggested

that the anti-proliferative effects seen in the MDA-MB-231 transfectant may be due to an alteration in growth factor expression. Alternatively, Lottering and co-workers (1992) have suggested that growth inhibition by oestrogen could be the result of enhanced production of 2-methoxy-17 β-oestradiol, a product of E_2 metabolism, which may be responsible for the cytotoxic effects seen with pharmacological concentrations of E_2 (Seegers *et al.*, 1989).

Previous reports suggest that oestrogens stimulate growth proliferation by shortening both G_1 and S-phases (reviewed by Sutherland *et al.*, 1983), and thus reducing the doubling time of the cells. Cell cycle measurements performed in the present study did not reveal any large differences between the distribution of cells in the parental PEO1 line following oestrogen treatment and the $PEO1^{CDDP}$ cell line, over a six day period. Slightly lower numbers of oestrogen-treated PEO1 cells were seen in G_0/G_1, and higher numbers were observed in S-phase and to a lesser extent in G_2/M as compared to untreated cells, but these differences were minimal and were also observed in oestrogen-treated $PEO1^{CDDP}$ cells. Notably, there were far greater numbers of PEO1 and $PEO1^{CDDP}$ cells in S-phase from both treatment groups than in the other cell lines. Reddel and Sutherland, (1987) reported a large decrease of cells in G_1 following high doses of diethylstilbestrol (2.5×10^{-6}M) or E_2 (5×10^{-6}M) for 48 hours, and accumulation of cells in the S and G_2/M phases of the cell cycle in both MCF-7 and T47-D cells, with the appearance of polyploid cells. A similar finding, which was also probably a consequence of high concentrations of E_2, was reported in ER-positive breast xenografts which were growth inhibited by E_2 at serum concentrations of approximately 10^{-7}M (Brünner *et al.*, 1983). No accumulation of treated $PEO1^{CDDP}$

cells was observed in G_2/M, and this suggests that the growth inhibition by E_2 in PEO1^{CDDP} is through another mechanism than that observed with pharmacological concentrations of oestrogens.

When MCF-7 cells are exposed to the lower concentration of 10^{-8}M E_2, a similar reduction in G_1 and increase in the S-phase population is seen although the percentage of cells in G_2/M remains steady, (Jiang and Jordan, 1992). Weichselbaum *et al.*, (1978) also reported an increase in the numbers of MCF-7 cells in S-phase after treatment with 10^{-9}M E_2 during a 30 hour period, but noted a decrease in the S-phase with a higher dose (10^{-7}M). Bezwoda and Meyer (1990) reported an increase of MCF-7 cells in S-phase after 48 and 72 hours of treatment but not at 24 or 96 hours. All these studies note large differences in the cell populations between control and treated cells unlike those noted here, in both the PEO1 pair of lines and the other ovarian cell lines examined. These studies suggest that further detailed examination of cell cycle data by examining earlier time points may reveal differences between cell cycle kinetics both in PEO1 and PEO1^{CDDP} cells, and the PEO4 and PEO14 ovarian cell lines. At present there appears to be little published literature on cell cycle analysis in other ovarian carcinoma cell lines.

The largest cell cycle changes were noted in the ZR-75-1 breast cell line. The percentage of oestrogen treated cells in G_2/M was much lower than control cells after six days, and this was concomitant with higher levels of treated cells in S and G_1. Similar but less pronounced differences were noted after three days. This increase in the S-phase population is consistent with the MCF-7 cell line data discussed above,

although these report a decrease in the percentage of treated cells in G_0/G_1. Recent data from Launoit *et al.*, (1991) showed an initial decrease in cells in G_1 following exposure of ZR-75-1 cells to 10^{-10}M E_2 for 24 hours. After further exposure, the number of cells increased again and stayed constant, suggesting that certain changes in the cell cycle in this cell line may be apparent earlier than assayed in this study.

Growth inhibition by oestrogen was observed when the PEO4 cell line was grown as a xenograft in nude mice. The mean residual volume of tumours which were not exposed to an exogenous 17 β-oestradiol pellet increased over a 75 day period but the volume of treated tumours virtually remained static. This study was carried out in mice which were not ovariectomised, so both control and treated groups were exposed to background levels of endogeneously produced oestrogens. PEO4 xenografts are growth inhibited in ovariectomised mice, compared to growth of xenografts in intact mice (Langdon, *et al.*, 1993) and this is consistent with a report by Sawada *et al.*, (1990) who noted decreased growth of the ER-positive OXA-5 ovarian xenograft in untreated male mice as compared to oestrogen-treated animals. Growth of PEO4 xenografts in ovariectomised mice supplemented with oestrogen is also inhibited compared to that seen in unsupplemented intact animals (Langdon *et al.*, 1993) and it is possible, therefore that endogenous oestrogen levels are sufficient for growth of PEO4 xenografts, whereas additional oestrogen supplement raises concentrations to a level which is inhibitory, similar to observations *in vitro* with high concentrations of oestrogen. This is borne out by observations that serum oestrogen concentrations in ovariectomised mice have been shown to be similar to that of male mice (approximately half that seen in intact mice), but levels are raised approximately

10-fold in both normal and ovariectomised mice with 17 β-oestradiol implants (Langdon *et al.*, 1993). Brünner *et al.* (1983) also reported that growth of T61 breast carcinoma xenografts was oestrogen inhibited.

HOX-60, an ER-negative xenograft, was not growth inhibited by treatment with oestrogen, and therefore it is unlikely that the effects on the PEO4 xenograft are due to general cytotoxicity. Alternatively, oestrogen may be influencing the action of other factors such as gonadotropins present in the *in vivo* situation which may override stimulatory effects on growth. The endogenous oestrogen levels in the mice were not sufficient to stimulate growth of the ER-positive ZR-75-1 breast carcinoma model which requires exogenous oestrogen (Osborne *et al.*, 1985).

4.2 Effects of oestrogen on oestrogen and progesterone receptor levels

In clinical studies, Clarke and McGuire, (1989) have reported ER to be present in 50-85% of all breast cancers, assuming a value of greater than 3 fmol/mg protein to be positive. ER expression is now widely used to predict the responsiveness of breast tumours to hormonal therapy, however about half of ER-positive tumours do not respond to treatment, suggesting that ER's in these cases are either not functional, or other signalling pathways are dominant. Further markers of oestrogen sensitivity have been sought. Progesterone receptors (PR) are induced in response to oestrogen (Horwitz and McGuire, 1978a) and are now used in conjunction with ER as a predictive marker in breast cancer. Tumours expressing both receptor types have a

70-75% response rate whereas less than 10% of tumours without ER respond to treatment (Brooks *et al.*, 1980).

A summary of receptor data from a number of studies reviewed by Slotman and Rao, (1988) suggests that 63% of ovarian tumours contain ER while PR are present in 48%. However, very little experimental data concerning these receptors exists, partly due to the paucity of receptor-positive cell lines, as explained before.

Levels of ER decreased in response to oestrogen in PEO1, PEO4, PEO6 and $PEO1^{CDDP}$ (the ovarian cell lines with substantial levels of the receptor), and also in the ER-positive ZR-75-1 breast line. This observation is compatible with the idea that oestrogen is acting through the ER, which is rapidly processed once occupied with ligand (Horwitz and McGuire, 1978b, Saceda *et al.*, 1988, Berkenstam *et al.*, 1989), and is similar to responses previously noted in breast cancer cell lines. Levels of ER in the PEA1 and PEA2 cell lines were near background and apparently unaffected by the addition of oestrogen. Although these cell lines are ER-positive, levels are low, and probably insufficient to elicit a mitogenic response. This is also consistent with the observation that oestrogen does not elicit a growth response with these two lines, unlike the cell lines with higher levels of ER. Likewise no change was observed in ER levels in the ER-negative PEO14, PEO16 and PEO23, values remained at the limit of detection.

As might be anticipated, oestrogen induces levels of PR in the PEO1, PEO4 and PEO6 cell lines, although it is doubtful whether the change is meaningful in PEO1, these values being at the limit of detection. A similar observation was noted in

ZR-75-1 breast cells, and although PR levels were far greater than those seen with the ER-positive ovarian cell lines, there was a similar fold induction in both ZR-75-1 and PEO6. These observations contrast with the previous findings of Nash *et al.,* (1989a) who despite observing a growth modulation by oestrogen, did not see PR induction in PEO4. This might be due to differing assay procedures, (Nash used sucrose density gradient analysis) or culture conditions. PR have also been shown to be induced in response to oestrogen in the ER-positive NIH:OVCAR-3 cell line (Hamilton *et al.*, 1984), which is growth stimulated by oestrogen.

No induction of PR expression was noted in either PEA1 or PEA2, or the $PEO1^{CDDP}$ cell lines. This is not unexpected for PEA1 and PEA2 if an increase in PR expression is indicative of oestrogen sensitivity; these two show no growth response to 17 β-oestradiol, or down regulation of ER. However, this is not true for the platinum-resistant cell line, where oestrogen causes a growth response, albeit an inhibition rather than a stimulation. As ER levels are reduced in response to oestrogen this indicates a direct effect of oestrogen acting through the ER. It is therefore possible that certain oestrogenic effects such as PR induction are only associated with a growth stimulation. However, PR levels are also induced in the ER-positive PEO4 xenograft which is also growth inhibited by oestrogen. The ER-negative HOX60 ovarian xenograft did not display any significant variation in either ER or PR levels, suggesting that 17 β-oestradiol again has a direct effect on PEO4 cells in the *in vivo* model, mediated through the ER, which is downregulated. These findings together with the previous data on the NIH:OVCAR-3 cell line (Hamilton *et al.* 1984) suggest that PR induction is an indicator of oestrogen sensitivity but not necessarily growth

stimulation. However in addition, as shown by the results obtained with the PEO1 cell line, a lack of significant PR induction does not necessarily indicate that a cell type is not hormonally sensitive.

In the PEO1^{CDDP} drug resistant model, oestrogen effects also do not appear to be coupled to an alteration in PR expression, values in both control and treated groups being at the limit of detection. It seems unlikely that this is a result of cisplatin selecting out a subpopulation of resistant cells with uncoupled PR responses as PR values were similar to the levels found in the parent PEO1 line, and thus these responses may be inherent to this line. Jiang and Jordan, (1992) previously noted that subclones of MCF-7 produce low levels of PR in response to oestrogen. However, the PEO4 cell line in which PR levels were induced by oestrogen was derived from the same patient as PEO1 but at a later stage in the progression of the disease following the onset of drug resistance. This model may represent a drug-selected subpopulation of cells demonstrating a different phenotype to PEO1, in which oestrogen effects are coupled to PR induction, or may be representative of a more advanced stage of the disease. As PEO1 is not a clonal line, the PEO4 cell phenotype may have been masked by a larger population of cells, although the observations with PEO1^{CDDP} cell line suggest that exposure to a selective pressure such as cisplatin does not favour the predominance of the PEO4 phenotype. Alternatively, it is possible that repeated culture of the PEO1 cell line *in vitro* has lead to the selection of a particular population of cells with uncoupled PR responses, from which the PEO1^{CDDP} cell line was derived, whereas, the PEO4 cell line model may be more representative of the

original tumour phenotype. This is readily testable by looking at oestrogen responses in early passage PEO1 cells.

Observations with these cell line models suggest that there is a need to delineate further markers of hormonal sensitivity, since lack of PR expression is not necessarily a sign of low hormonal responsiveness. This is also reflected clinically in breast tumours. A recent trial of patients with ER-positive, PR-negative (< 10 fmol/mg protein) metastatic breast cancer demonstrated a 43% response rate to tamoxifen (Ravdin *et al.*, 1992). However, this study confirmed the value of elevated PR levels and emphasised the importance of use other predictive factors in conjunction with steroid receptor status.

4.3 Effects of oestrogen on pS2 expression

Several oestrogen-inducible markers have been described which may act as predictors of hormonal responsiveness in breast cancer. Amongst these is the pS2 protein, of unknown function, which is secreted by the ER-positive breast cancer cell line, MCF-7 (Nunez *et al.*, 1987). A number of reports have shown it to be positively correlated with the presence of ER (Rio *et al.*, 1987, Schwartz *et al.*, 1991, Henry, 1991a, Thompson *et al.*, 1993) and overall survival in breast tumours (Gion *et al.*, 1993, Besse *et al.*, 1994) although this is not always the case (Wysocki *et al.*, 1994).

There have been few reports of pS2 expression in ovarian cancer, probably due to only a recent interest in hormonal regulation of this disease and none of these have investigated its regulation in cell line models. pS2 expression and its possible

modulation by oestrogen was examined in five of the ovarian cell lines with varying ER levels. However, only a small level of staining (< 3%) was observed in both control and treated groups after three or six days in experimental conditions. Some small changes were observed in staining intensity between control and treated PEO1 and PEA1 cells but as these values were just above the background level of 1.0, they are unlikely to represent a major effect. In contrast, pS2 expression was seen in ER-positive ZR-75-1 breast cells, similar to previous reports by Darbre and Daly, (1990). Moreover, levels were increased about 3 fold after oestrogen treatment.

These findings are in agreement with a study by Foekens *et al.*, (1990b) who found no pS2 expression in a sample of 26 advanced ovarian tumours, and together suggest that pS2 may have no utility as a predictive marker in ovarian cancer, unlike the situation for breast cancer. Interestingly, pS2 staining has not been found in a variety of normal tissues including ovary and endometrium (Rio *et al.*, 1988b).

However, reports of positive expression in ovarian cancer have been recorded. Wysocki *et al.*, (1990) detected pS2 mRNA in 6 out of 29 primary ovarian carcinomas. Expression appeared to be correlated with histological subtype, as four of these tumours were mucinous cystadenocarcinomas, this group exhibiting a much stronger pS2 band than the one serous cystadenocarcinoma which was positive. Tumour numbers were not high enough for this to prove statistically significant. A similar and significant result was obtained by Henry *et al.*, (1991b) who found 6 out of 9 pS2-positive ovarian tumours were of the mucinous subtype, and a more recent study by Dante *et al.*, (1994) has also confirmed a relationship between the mucinous

subclass and pS2 expression. This group also examined pS2 expression in ovarian cystadenomas, and again found the positive correlation with mucinous histotype. No expression was observed in serous cystadenomas.

Expression of pS2 in Wysocki's study did not appear to correlate with ER which is in conflict with the majority of breast cancer data. Dante also suggested that pS2 expression was not oestrogen-dependent having found pS2 mRNA in oestradiol-free ascitic fluid from mucinous cystadenomas. It is possible that in these tissues pS2 expression is independent of oestrogen regulation. pS2 levels have been shown to be induced by a number of factors including epidermal growth factor (Nunez *et al.*, 1989), and so expression may regulated differently in individual tissues. Some evidence from breast cancer also indicates that pS2 may be hormone independent. Johnston *et al.*, (1995) found that a set of breast tumours which had progressed on tamoxifen therapy were ER-negative, but still expressed pS2 and PR, possibly due to elevated levels of a variant form of ER, and Brünner *et al.*, (1993a) showed that a series of hormone independent MCF-7 sublines had high pS2 expression compared to parental cells. Evidence suggests that pS2 may be a growth factor (Rio *et al.*, 1987) and its constitutive expression may be important in oestrogen independent growth. The presence of pS2 may also be related to the production of mucin. In addition to Dante's and Wysocki's findings, high levels of pS2 have been found in stomach mucosal cells (Rio *et al.*, 1988b), and strong positivity is found in mucinous (colloid) breast carcinomas (Henry *et al.*, 1991a).

The ovarian cancer cell lines in this study were derived from serous ovarian carcinomas, and are representative of the majority of diagnosed cases which are of this histological category. There is no mention of the ovarian tumours' histology in Foeken's report (1990b) so no conclusions can be drawn as to the lack of expression. Further studies are needed to help define the function of the pS2 protein. It has some characteristics similar to IGF-I and other small peptide growth factors (reviewed by Rio *et al.*, 1990), and has close homology with porcine pancreatic spasmolytic peptide which causes a growth stimulation in breast cancer cells (Hoosein *et al.*, 1989). However, Neri *et al.*, (1991), have suggested that pS2 may have a function other than as a growth regulator from studies which noted a lack of close association between pS2 expression and proliferation in MCF-7 cells. So far in ovarian cancer there is no evidence that it may serve as a predictive marker for hormonal sensitivity, but it may have limited use as a specific marker for mucinous carcinomas.

4.4 HSP27 expression and its modulation by oestrogen

Interest in HSP27 has mounted over the last ten years as evidence accumulates to support its role as a predictive marker in breast and endometrial cancer (reviewed by Ciocca *et al.*, 1993). Furthermore, recent data suggest an involvement in drug resistance (Oesterreich *et al.*, 1993). This study is the first report of HSP27 expression in ovarian cancer. In order to determine a relationship between HSP27 levels and hormonal responsiveness, expression was investigated in routinely cultured cells and after oestrogen treatment.

In the majority of cell lines HSP27 expression correlated with the presence of ER and oestrogen responsiveness, levels being highest in the PEO1, PEO4 and PEO6 cell lines which have a moderate to high ER status and are growth stimulated by E_2. This is similar to data obtained with breast tumours (King *et al.*, 1987b, Dunn *et al.*, 1993). However, only a low level of expression was observed in the cisplatin resistant $PEO1^{CDDP}$ cell line although this expresses similar levels of ER to that of the parental PEO1 cell line. This may be a consequence of drug selection, however, when grown in 5% dcsFCS, HSP27 expression in $PEO1^{CDDP}$ is comparable with PEO4. Whelan and Hill, (1993) have shown that ER-positive MCF-7 cells which are selected for resistance to vincristine lose expression of HSP27 and pS2. However, unlike $PEO1^{CDDP}$ cells they also show no detectable ER expression either. These changes were accompanied by overexpression of the P-glycoprotein and a classical multi-drug resistance phenotype, which is not involved in cisplatin resistance. It is feasible, however, that downregulation of pS2 and HSP27 expression occurred as a result of loss of the ER in these cells, and not as a direct consequence of drug resistance or MDR1 expression.

However, there is evidence to suggest that HSP27 may be important in resistance to certain types of drugs. Huot *et al.*, (1991) have shown that Chinese hamster ovary cells which overexpress transfected human HSP27 are resistant to doxorubicin, colchicine and vincristine but not 5-flurouracil or the nitrosoureas. In addition, heat shock induction of HSP27 in MCF-7 and MDA-MB-231 cells also renders them resistant to doxorubicin, although not to other agents (Oesterreich *et al.*, 1993).

HSP27 expression may have relevance to *in vivo* derived clinical resistance in ovarian cancer. The cell lines, PEO1, PEO4 and PEO6 were all derived from the same patient but levels of HSP27 protein in the PEO4 and PEO6 cell lines which were derived after onset of resistance were two to four fold higher than PEO1. Moreover, HSP27 levels are significantly higher in primary ovarian tumours which progress on therapy, as compared to ones which are static or responsive (Langdon, personal communication).

Positive associations between ER and HSP27 expression were also observed *in vivo*, when levels of the protein were examined in ER-positive ZR-75-1 breast and PEO4 ovarian cell lines grown as xenografts. Both these models expressed higher levels of HSP27 than the ER-negative HOX60 and T1068 xenografts. Expression *in vivo* appeared to be comparable with levels observed in the *in vitro* cultured lines, although the higher expression in the ZR-75-1 xenograft may reflect higher levels of oestrogen administered to the tumour in the *in vivo* situation (as a result of an oestrogen pellet being necessary to sustain growth of the xenograft).

A further connection between ER and HSP27 expression was noted when levels of the protein were examined after cells had been treated with oestrogen. Differences in expression were noted only in the ER-positive cell lines, although levels were downregulated in the PEO4 and $PEO1^{CDDP}$ lines but upregulated in ZR-75-1. This latter response is in line with previous reports which have shown HSP27 to be upregulated both at the mRNA and protein level in MCF-7 and ZR-75-1 cells (Fuqua *et al.*, 1989, Ciocca *et al.*, 1983 Ciocca *et al.*, 1992). HSP27 may be differentially

regulated in breast and ovary, however, an upward trend in expression was also noted in the PEO1 cell line after E_2 treatment and although this proved non-significant it may delineate certain differences in the oestrogen response pathway between itself and the PEO4 and PEO1^{CDDP} cell lines. Expression was not significantly altered by oestrogen treatment in the *in vivo* PEO4 model. However, as these experiments were performed in non-ovariectomised animals, circulating oestrogens may modulate HSP27 expression, such that no further change in levels are seen in xenografts exposed to E_2 supplement.

4.5 Effect of oestrogen on growth factor expression

The exact mechanism by which oestrogen regulates growth in ovarian cancer is still undefined although characterisation of oestrogen response pathways in breast cancer suggest that it may mediate part of its action through the modulation of growth factors in an autocrine or paracrine manner. Expression of TGF-α and IGF-I are both increased by oestrogen treatment in MCF-7 cells (Bates *et al.*, 1988, Huff *et al.*, 1988). EGF receptors are commonly expressed in ovarian cancer (Bauknecht *et al.*, 1988, Battaglia *et al.*, 1989, van der Burg *et al.*, 1993) and increasing evidence is revealing the importance of autocrine mechanisms in the growth of this carcinoma.

The production of TGF-α and EGF by oestrogen-treated and untreated cells grown in serum-free media was measured by radioimmunoassay. Production of TGF-α was detected in the conditioned media from all of the ovarian cell lines (PEO1, PEO4, PEO1^{CDDP} and PEO14) but was highest in the PEO1^{CDDP} line. Leake *et al.*, (1995)

have recently shown that the TGF-α levels found in cytosolic fractions of PEO1^{CDDP} are also greater than in PEO1 and PEO4 cytosols, fitting in with observations in this thesis. Although the concentrations of TGF-α detected were low, they were similar to the lower end of a range of levels detected in a series of ovarian cell lines described by Stromberg *et al.*, (1992). Values were also uncorrected for assay efficiency and so are likely to be lower than actual levels of secreted TGF-α. Additionally, it is possible that levels of secreted TGF-α may be an underestimate of endogenously produced growth factor since TGF-α may exist as a membrane-bound biologically active precursor molecule (Wong *et al.*, 1989, Rainer *et al.*, 1991). Consistent with previous reports of oestrogen modulation of TGF-α in ER-positive breast carcinoma lines (Dickson *et al.*, 1986, Bates *et al.*, 1988, Kenney *et al.*, 1993), an increase in TGF-α expression was noted in both ER-positive PEO4 and PEO1^{CDDP} lines which were exposed to oestrogen. A similar increase was noted in the ER-positive PEO1 cell line although this was at borderline significance ($p=0.05$), but no change was noted in expression in the oestrogen unresponsive ER-negative PEO14 cell line. When normalised against cell number the difference in expression between treated and untreated cells is still observed and shows that the increase in TGF-α secretion is not purely an effect of increased cell number, and in fact there is slightly reduced growth of cells in serum-free conditions.

Crew *et al.*, (1992a) have shown growth stimulatory effects of TGF-α in the PEO1 and PEO4 cell lines, and taking this data into account there is a strong suggestion that TGF-α may play an important role in the mediation of oestrogen-stimulated growth

responses in ER-positive ovarian cell lines. Growth of the ER-negative PEO14 was also shown to be stimulated by TGF-α and this is consistent with the fact that all three cell lines possess EGF receptors (Crew *et al.*, 1992a). A low level of TGF-α was detected in the culture medium of PEO14 and suggests that this growth factor may also be involved in the growth regulation of this line, in an oestrogen-independent mechanism. There are an increasing number of reports in the literature to support the role of TGF-α in the growth regulation of ovarian cancer. Several groups have noted the growth stimulatory action of TGF- α in ovarian cancer cell lines (Morishige *et al.*, 1991b, Zhou *et al.*, 1992, Stromberg *et al.*, 1992). Moreover, Stromberg *et al.*, (1992) and Morishige *et al.*, (1991b) have both reported the production of TGF-α in EGF-R positive ovarian cancer cell lines and growth inhibition with neutralizing antibodies against TGF-α. Similar observations are noted with primary human ovarian cancer models and in xenografts providing further evidence for an autocrine growth mechanism in ovarian cancer (Kurachi *et al.*, 1991, Morishige *et al.*, 1991a).

There was no change in the expression of TGF-α in ER-positive ZR-75-1 breast cells which had been oestrogen-treated, although this line produced the greatest detectable amount of the growth factor, twice as much as $PEO1^{CDDP}$. Expression of TGF-α mRNA has previously been described in ZR-75-1, although at a level less than that seen in ER-negative MDA-MB-231 breast cells (Bates *et al.*, 1988) who noted that basal expression of TGF-α appears to be unrelated to ER status. An increase in TGF-α expression by oestrogen in a clone of ZR-75-1 has been reported by Dickson

et al., (1986), although the level of induction was lower than that seen in MCF-7 cells. Kenney *et al.*, (1993) have suggested that oestrogen-modulation of TGF-α is an important mechanism in the growth of ZR-75-1 cells by showing that oestrogen-stimulated growth is inhibited by infection with an antisense TGF-α retroviral vector. Why no modulation of TGF-α expression was observed in the present study is unclear, although clonal selection can not be ruled out.

No EGF production could be detected in conditioned media from any of the cell lines either treated or untreated, and it is possible that the radioimmunoassay used was not sensitive enough to detect EGF levels present, operating in the nanomolar range. As such, EGF may have been detected if further concentration of conditioned media (greater than x100) was carried out. However, EGF may be only a minor element in growth regulation of these ER-postitive cell lines, with the main effector being TGF-α. Crew *et al.*, (1992a) have shown that PEO1, PEO4, and PEO14 are also growth stimulated by EGF to a similar degree as TGF-α. Similar findings were described by Berchuck *et al.*, (1990b) who found that although the OVCAR 429 and 433 cell lines showed a growth response to EGF, there was no EGF-like activity in the conditioned medium from these cells. Evidence of a TGF-α-predominant growth regulation pathway was also noted by Morishige and co-workers, (1991b) who found no EGF expression by either northern blotting or immunohistochemistry in the SHIN-3 ovarian carcinoma cell line, but detected TGF-α and EGFR expression by both techniques. Fitting in with this observation, they also found that monoclonal antibodies directed against EGF did not inhibit the growth of SHIN-3 cells, in

contrast to significant growth inhibitions observed with antibodies against TGF-α or EGF-R. Leake *et al.*, (1995) found that only a minority of ovarian tumours contained measurable levels of EGF, whereas over 80% contained TGF-α, implying that EGF may be of less importance in the growth regulation of this cancer.

The evidence that oestrogen produces an increase in TGF-α production in the $PEO1^{CDDP}$ cell line is interesting in the light of the fact that this is concomitant with growth inhibition, suggesting perhaps that in this cell line the normal oestrogen regulated TGF-α pathway is abrogated, possibly by factors downstream of receptor activation. Oestrogen induces an increase in TGF-α mRNA expression in MDA-MB-231 cells stably transfected with a wild type ER expression vector, and this is also associated with a growth inhibition (Jeng *et al.*, 1994). More recent work has shown that addition of TGF-α to $PEO1^{CDDP}$ cells also causes growth inhibition, in contrast to the parental line in which there is growth stimulation (Simpson, personal communication). Furthermore, using a neutralising monoclonal directed against the EGF receptor, these effects on growth can be partially reduced in the order of 40-80%. There is some evidence to suggest a link between growth factor receptor expression and drug sensitivity. Christen *et al.*, (1990) have reported that treatment of two human ovarian carcinoma cell lines with EGF sensitises the cells to cisplatin, and sensitization is both dependent on EGF-R numbers and concentration in mouse fibroblasts expressing transfected EGF-R. EGF did not enhance the cisplatin sensitivity of CDDP-resistant 2008 ovarian cells which express 3 fold fewer EGF-R than sensitive 2008 cells, and they suggested that the resistant phenotype was associated with a defect in the signal transduction pathway. In contrast, increased

levels of EGF-R have been found in multi-drug resistant chinese hamster and mouse tumour cell lines (Meyers *et al.*, 1986). In addition, there is data to support an association between drug resistance and c-*erb*B2 expression. Overexpression of this receptor is known to be associated with poor outcome in ovarian cancer (Slamon *et al.*, 1989, Berchuck *et al.*, 1990a) and recent work shows that antibodies against c-*erb*B2 can block DNA repair after cisplatin treatment, and increase the cisplatin sensitivity of both SKBR3 cells which constitutively overexpress c-*erb*B2, and 2008/C13 CDDP-resistant ovarian cancer cells which overexpress c-*erb*B2 as a result of infection with a retroviral construct containing full length human c-*erb*B2 (Pietras *et al.*, 1994). These data do not suggest that overexpression of c-*erb*B2 can cause increased drug resistance as there was no difference in the cisplatin sensitivity of C13 vector alone controls and c-*erb*B2 overexpressing cells, although transfection of MCF-7 breast cells with c-*erb*B2 does decreases sensitivity to cisplatin (and tamoxifen) (Benz *et al.*, 1991). Langton-Webster *et al.*, (1994) have shown that when SKOV-3 ovarian carcinoma cells which constitutively overexpress c-*erb*B2 are made resistant to cisplatin there is a reduction in c-*erb*B2 expression, and therefore it may be interesting to evaluate levels of c-*erb*B2 in $PEO1^{CDDP}$ as compared to PEO1 to determine whether there is decreased c-*erb*B2 expression in the resistant subline.

There are varying reports of TGF-α and EGF-induced cell inhibition, and several studies suggest that the action of EGF or TGF-α on cells may depend on the levels of c-*erb*B2 receptor present. Mills *et al.*, (1992) have shown that both these growth factors increase proliferation in two ovarian cancer cell lines (OCC1 and OCC3) which overexpress c-*erb*B2, whereas there is decreased growth in two other lines

(OCC2 and HEY) which expresss normal levels of the c-*erb*B2 receptor. C-*erb*B2 is thought to potentiate the EGF signal and becomes rapidly phosphorylated when certain cell types are treated with the EGF (GrausPorta *et al.*, 1995). When tyrosine kinase activity was assessed in the OCC1 and HEY ovarian cell lines, it was noted that the level of tyrosine phosphorylation of the EGF-R was similar in the two lines, but c-*erb*B2 phosphorylation was observed only in the OCC1 cells, consistent with the observation that OCC1 cells have higher levels of c-*erb*B2 as compared with the HEY cell line (Mills *et al.*, 1992). Other workers have shown that EGF has converse effects on cell proliferation depending on the level of EGF receptors. Addition of EGF to tumour lines A431 and MDA-MB-486 which overexpress EGF-R cause a growth inhibition, whereas other cell lines expressing moderate levels of EGF-R are growth stimulated (Filmus *et al.*, 1985, Gill and Lazar, 1981, Barnes, 1982). The growth inhibition in A431 has been shown to be associated with a sustained increase in p21(WAF-1/cip1) and reduced cyclin-dependent kinase-2 (CDK2) activity associated with a G_1 arrest, (Fan *et al.*, 1995). Gulli *et al.*, (1996) have suggested that this growth inhibition is dependent on the level of EGF-R autophosphorylation in A431: high levels being associated with growth inhibition, whereas lower levels, a result of reduced availability of ligand, are associated with a growth proliferation. Additionally, Chen and Lin, (1993) have previously reported that growth inhibition in A431 is associated with increased tyrosine kinase activity as compared to the EGF growth-stimulated A431-4 subline which expresses lower levels of EGF-R. Interestingly, recent data shows that tyrosine kinase activity is highest in the $PEO1^{CDDP}$ cell line as compared to PEO1 or PEO4, is associated with raised levels of

TGF-α, and is further increased by 17 β-oestradiol (Leake *et al.*, 1995), although the level of EGF-R expression in $PEO1^{CDDP}$ has not been determined. Potentially, an abnormal level of tyrosine kinase activity induced by TGF-α may alter the normal signal transduction pathway in $PEO1^{CDDP}$, possibly bringing into effect elements of a negative feedback pathway involving other growth factors which may inhibit growth.

A number of other growth factors are implicated in the progression of ovarian cancer and amongst these, the importance of the IGF family of growth factors in ovarian cancer is now emerging. IGF's are major determinants of paracrine control of normal ovarian function, but less is known about their role in ovarian cancer, and whether or not they are influenced to any extent by hormones such as oestrogen. When levels of IGF-I receptors were measured in PEO1 and PEO4 cell lines, a small but significant increase in expression levels was noted in PEO4 cells which had been exposed to oestrogen treatment as compared to untreated controls. A similar response was observed in PEO1. This finding is consistent with reports in breast cancer cell lines where IGF-I receptors are also up-regulated by oestrogen (Stewart *et al.*, 1990), and a previous study by Wimalasena *et al.*, (1993) who saw an increase in IGF-I receptors after oestrogen treatment in an ovarian cancer cell line. It has been recently confirmed that both the PEO1 and PEO4 cell lines express mRNA for IGF-I, are stimulated by the growth factor, and also contain receptors for IGF-II (Bartlett *et al.*, 1995). In addition, both the cell lines are stimulated by insulin, but not IGF-II, despite possessing receptors for the latter. This data cumulatively points to IGF-I being a regulator of growth in PEO1 and PEO4, and oestrogen may possibly effect growth responses through modulation of these factors.

IGF-I receptors have been shown to be expressed in a number of ovarian cell lines and several are growth stimulated by IGF-I (Yee *et al.*, 1991a, Resnicoff *et al.*, 1993), however data on the effects of oestrogen modulation are sparse. Krywicki *et al.* (1993) have shown that oestrogen can modulate the expression of certain IGF binding proteins (IGFBP's) in PEO4; mRNA levels for IGFBP-3 are decreased, as are IGFBP-2, -4 and -6 although to a lesser extent, whereas IGFBP-5 levels are increased. However, they also showed that IGF-I mRNA was not altered by oestrogen. IGF's associate with IGFBP's and modulate the level of ligand/receptor interaction (De Mellow and Baxter, 1988) and it is possible that reduced levels of binding proteins after oestrogen treatment in PEO4 may make the cells more responsive to IGF-I (Krywicki *et al.*, 1993), and this may agree with an increase in the levels of IGF-I receptors following oestrogen treatment. In MCF-7 breast cancer cells, 17 β-oestradiol increases the sensitivity of the cells to the growth stimulatory effects of IGF-I (Thorsen *et al.*, 1992). This effect has also been noted by Wimalasena *et al.*, (1993) who showed that oestrogen could enhance the growth effects of IGF-I in ovarian cancer cells. IGFBP-2 may be one of the more important regulators of IGF activity in ovarian cancer. Krywicki *et al.*, (1993) showed it to be preferentially expressed in a series of ovarian tumour samples, and it is elevated in cyst fluid and serum from patients with malignant ovarian tumours as compared with benign neoplasms (Karasik *et al.*, 1994, Kanety *et al.*, 1996). IGFBP-2 is induced by oestrogen in ER-positive breast cancer cells (Yee *et al.*, 1991b) and a correlation between oestrogen and IGFBP-2 content of cyst fluids from ovarian tumours has been noted (Karasik *et al.* 1994).

The effect of steroid hormones on the IGF family in breast cancer is better understood. Oestrogen up-regulates the IGF-I receptor in breast cancer cells (Stewart *et al.*, 1990, van den Berg *et al.*, 1996) and a positive correlation between ER and IGF-I R has been shown (Pekonen *et al.*, 1988, Railo *et al.*, 1994). Oestrogen also induces the levels of IGF-II mRNA in the T47-D and MCF-7 breast cell lines (Myal *et al.*, 1984, Yee *et al.*, 1988). In addition it has been shown that anti-oestrogens can influence the levels of circulating IGF-I and expression of IGFBP's (Pratt and Pollak *et al.*, 1993, Lahti *et al.*, 1994). Levels of IGF's may be involved in the growth inhibition observed in T61 breast xenografts seen after treatment with both oestrogen and tamoxifen. Brünner *et al.*, (1993b) reported that IGF-II mRNA was down regulated by both treatments in the xenograft. A monoclonal antibody against the IGF-I receptor also inhibited growth, implicating IGF-II in the growth of the xenograft. As such, a change in IGF expression may also be partly responsible for the oestrogen-induced growth inhibition observed in $PEO1^{CDDP}$ cells.

4.6 Conclusions and Future Work

Data presented in this thesis have shown that the growth of *in vitro* and *in vivo* ovarian cell lines which possess moderate to high levels of oestrogen receptors can be modulated by oestrogen, whereas ovarian cell lines which express lower levels of oestrogen receptor (< 20 fmol/mg protein) are not. These effects on growth may be mediated through the regulation of TGF-α and members of the IGF family, possibly in an autocrine and/or paracrine pathway (see Figure 4.1). Furthermore, these growth

effects are associated with increased levels of other proteins, PR and HSP27 which are putative markers of hormonal sensitivity in breast cancer.

As the majority of ovarian tumours possess ER, it seems plausible that anti-oestrogen therapy may be of benefit in the treatment of this cancer, however there have been only a limited number of trials conducted so far. This study suggests that a certain subset of patients with moderate to high levels of ER may be responsive to treatment strategies involving the anti-oestrogen tamoxifen, or one of the newer pure anti-oestrogens. In addition, HSP27 and PR may have value as predictive markers of endocrine response in ovarian cancer similar to reports in breast and endometrial carcinoma, and aid selection of treatment strategy. Alternatively, drugs directed against the downstream target of oestrogen, for example growth factor receptors, may also be of benefit.

A clinical study investigating the use of tamoxifen in the treatment of ER-positive ovarian tumours will soon be initiated in the Edinburgh Medical Oncology Unit. Based on some of the observations described in this thesis, patients will be selected for treatment on the basis of moderate-high levels of ER. Several markers which may be related to response will be measured and include PR, HSP27 and the EGFR.

Further work with the cisplatin resistant derivative of PEO1 will help determine the mechanisms whereby oestrogen elicits a growth inhibition, and answer several important questions which may have relevance for treatment of resistant tumours in the clinical setting. More detailed flow cytometric analysis may help elicit which cell cycle changes are occurring and whether this an increase in apoptosis or a reduction

in the mitotic index in this cell line. In addition, does tamoxifen or other anti-oestrogens reverse the effects of oestrogen, or also cause a growth inhibition? As oestrogen induces a growth inhibition in ER-transfected breast cells it may be of interest to see whether parallel changes occur in ovarian cancer. *In vivo* models derived from $PEO1^{CDDP}$ may also aid characterisation of this cell lines phenotype in a more realistic environment.

Evidence presented in this thesis suggests that the oestrogen-induced growth inhibition of $PEO1^{CDDP}$ is possibly due to a modified growth pathway involving one or more growth factors. If this model of drug resistance is typical of the clinical situation then it implies that care should be taken in future treatment strategies involving drug resistant tumours.

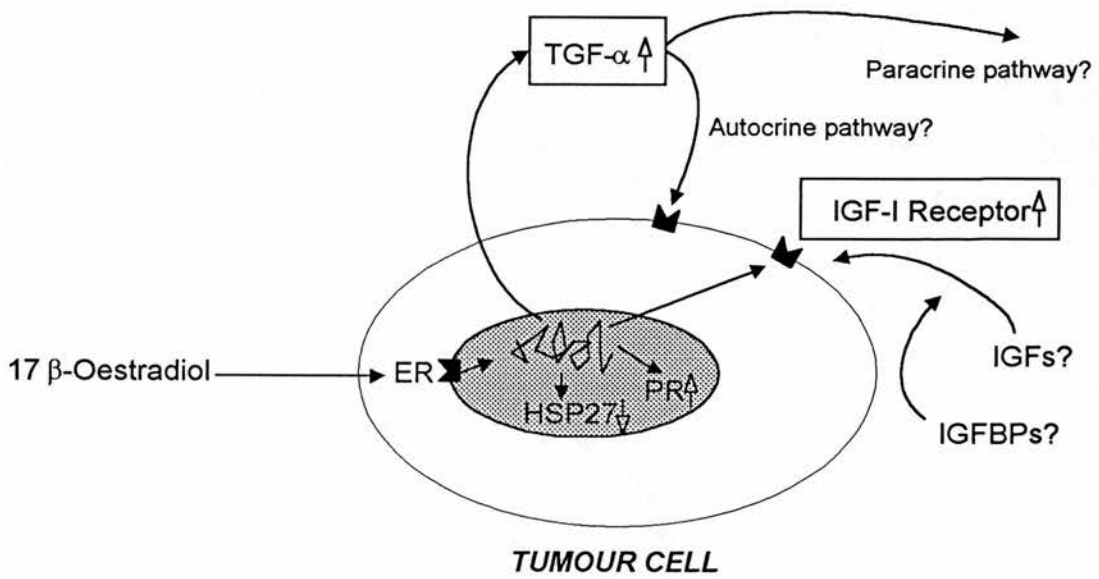


Figure 4.1

Schematic summarising the results in this thesis, showing the effects of oestrogen on the expression of certain proteins, and the possible pathways by which it may mediate its effects on growth in an ER-positive ovarian cancer cell.

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6. APPENDIX

Data from this thesis has been presented in the following abstracts and papers:

Papers

Langdon, S.P., Hirst, G.L., Miller, E.P., Hawkins, R.A., Tesdale, A.L., Smyth, J.F. and Miller, W.R. (1994) The regulation of growth and protein expression by estrogen *in vitro* : A study of 8 human ovarian carcinoma cell lines. *J Steroid Biochem Mol Biol* 50 No.3/4 131-135

Bartlett, J.M., Rabiasz, G.J., Scott, W.N., Langdon, S.P., Hirst, G.L., Lee, A.L., King, R.J., Smyth, J.F. and Miller, W.R. (1995) Growth control of human ovarian carcinoma cells by insulin-like growth factors. *Oncology Reports* 2 857-862

Langdon, S.P., Rabiasz, G.J., Hirst, G.L., King, R.J.B., Hawkins, R.A., Smyth, J.F. and Miller, W.R. (1995) Expression of the heat shock protein HSP27 in human ovarian cancer. *Clinical Cancer Res* 1 1603-1609

Abstracts

Hirst, G.L., Langdon, S.P., Miller, E.P., Tesdale, A.L., Hawkins, R.A., Smyth, J.F. and Miller, W.R. (1993) Oestrogen modulation of growth and protein expression in oestrogen receptor-positive ovarian carcinoma cell lines. *Br J Cancer* 67 suppXX 50

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The Regulation of Growth and Protein Expression by Estrogen *In Vitro*: A Study of 8 Human Ovarian Carcinoma Cell Lines

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The effects of 17 β-estradiol (E_2) on the growth and the levels of estrogen receptor (ER), progesterone receptor (PR) and pS2 protein were examined in a range of 8 ovarian carcinoma cell lines. E_2 stimulated growth of the 3 cell lines with an ER content of 80–220 fmol/mg protein but not the 5 cell lines with ER concentrations less than 20 fmol/mg protein. After exposure to E_2, ER concentration in 2 of the 3 responsive cell lines was decreased relative to untreated cells and in 2 lines, progesterone receptors were increased. No change in steroid receptor levels was observed in cell lines with low or negligible levels of receptors. The pS2 protein was not induced by E_2 in the 5 ovarian carcinoma cell lines examined. These results indicate that E_2 can stimulate the growth of some ER-positive ovarian carcinoma cells and that these effects may be associated with changes in the cellular levels of steroid hormone receptors.

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INTRODUCTION

The majority of human ovarian adenocarcinomas are estrogen-receptor (ER)- positive (reviewed in Slotman and Rao, 1988 [1]) and experimental evidence is accumulating to indicate that, at least a proportion of, these tumours are estrogen-responsive [2–5]. This is consistent with the clinical observation that a subset of ovarian cancer patients are responsive to the anti-estrogen tamoxifen [6–8]. To define those characteristics that determine sensitivity to estrogen (and therefore anti-estrogens) in this disease, we have developed a series of ovarian cancer cell line models [2, 9]. Using these models, we have previously demonstrated that growth of the ER-positive ovarian carcinoma line PE04 line is modulated by 17 β-estradiol (E_2) *in vitro* and *in vivo*, while growth of the ER-negative PE014 line is not [2, 3]. In addition to growth responses, we are also attempting to define proteins whose expression is under estrogen control. The identification of estrogen-regulated proteins should lead to an improved understanding of the mechanisms of estrogen (and anti-estrogens) in this disease and provide possible indicators of hormonal sensitivity in clinical specimens. In breast cancer, estrogen-regulated markers, which have been shown to have utility, include the progesterone receptor (PR) [10, 11], the pS2 (or pNR-2) protein [12, 13] and procathepsin D [14, 15]. This last protein has already been demonstrated as being increased in ER-positive ovarian cancer cells after exposure to E_2 [5, 16].

In the present study, we have investigated the ability of E_2 to influence the growth of a series of ovarian carcinoma cell lines with moderate–high levels of ER (PE04, PE01, PE06) and with low or negligible levels of ER (PEA1, PEA2, PE014, PE023, PE016) [2]. The levels of ER, PR and pS2 have been measured after culture in the absence and presence of E_2 treatment in these lines to determine whether these proteins are modulated and might function as markers of estrogen sensitivity. Comparisons are made with the ER-positive ZR-75-1 and ER-negative MDA-MB-231 breast carcinoma cell lines.

EXPERIMENTAL

Cell lines

The human ovarian carcinoma cell lines (PE01, PE04, PE06, PEA1, PEA2, PE014, PE023 and PE016) were established and characterized as described

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previously [9]. Cells were routinely maintained at 37°C in a humidified atmosphere of 5% CO_2 in air in RPMI 1640 (Gibco) containing 10% heat-inactivated foetal calf serum (FCS) and supplemented with streptomycin (100 μg/ml), penicillin (100 IU/ml) and glutamine (2 mM). The breast carcinoma cell lines (ZR-75-1 and MDA-MB-231) were maintained in DMEM (Gibco) containing FCS and the same additives as for the ovarian carcinoma lines.

Growth studies

Exponentially growing cells were harvested by trypsinisation and plated in 24-well plates (Falcon) at densities of 2.5–5 × 10^4 cells/well (4 wells/experimental condition) in RPMI 1640 containing phenol red and 10% FCS. After 24 h, to allow for attachment, the medium was removed and phenol red-free RPMI 1640 containing 5% charcoal-stripped FCS [17] was added. The cells were incubated for a further 24 h and the media were removed. RPMI 1640 containing 5% charcoal-stripped FCS, with or without E_2, at concentrations ranging from 10^{-12} to 10^{-5} M was added. Medium, with or without E_2, was replenished 3 days later. Cells were trypsinized from wells after a total of 6 days exposure and counted using a Coulter Counter.

Determination of ER and PR by enzyme-immunoassay

Mid-log phase cells growing in 175 cm^2 flasks were treated with or without 10^{-10} M E_2 for 6 days as described in the growth experiments. After 6 days treatment, medium was removed and cells were harvested by scraping. The contents from 4 identical flasks were pooled to give each pellet and these were stored at −80°C until use. Cell pellets (50–200 mg) were weighed and homogenized in buffer (10 mM Tris, 0.25 M sucrose, 1 mM ethylene diammine tetraacetate, pH 8.0, plus 1% v/v monothioglycerol and 10% v/v glycerol) as previously described [18, 19]. After centrifugation at 105,000 *g*, the supernatant cytosol was assayed using the ER-EIA or PR-EIA kits provided by Abbott Laboratories (Maidenhead, Berkshire, U.K.), according to the manufacturer's instructions [20]. The protein content of the cytosol was determined by the method of Bradford [21] and receptor concentrations were expressed as fmol/mg protein. We arbitrarily consider tissue/cells containing less than 5 fmol/mg protein as "receptor-negative".

Determination of pS2 by flow cytometry

Cells were plated at a density of 5–10 × 10^4/ml (2 ml/well) into 6-well plates (Falcon) and subjected to 3 or 6 days exposure to medium with or without 10^{-10} M E_2. pS2 was then detected by flow cytometry as follows. Cells were trypsinized, fixed in 70% ethanol at 4°C for 30 min and washed twice in PBS containing 5% FCS and 0.5% Tween 20 (wash buffer). Anti-pS2 antibody (100 μl; Histo-CIS, Gif-sur-Yvette Cedex, France) was diluted 1:2 in the above buffer on ice and added to the cells for 30 min. Cells were then washed in buffer. Sheep anti-mouse FITC conjugate (Sigma, U.K.) diluted 1:20 in buffer was added for 30 min to cells on ice. Cells were then washed once in buffer, then twice in PBS, resuspended in PBS and analysed on a FACScan flow cytometer (Becton Dickinson).

RESULTS

Effect of E_2 on growth

The 8 ovarian carcinoma cell lines were treated with E_2 at concentrations ranging from 10^{-12} to 10^{-5} M for 6 days. The 3 lines with moderate–high levels of ER (PE04, PE01 and PE06) were all growth stimulated by E_2 at concentrations between 10^{-12} and 10^{-7} M (Fig. 1). Growth stimulation was maximal at concentrations of 10^{-10} and 10^{-9} M E_2 and the former concentration was selected for the protein modulation experiments below. The growth of the other lines (PEA1, PEA2, PE014, PE023 and PE016) with low to zero levels of ER were unaffected by E_2 over this range of concentrations (data

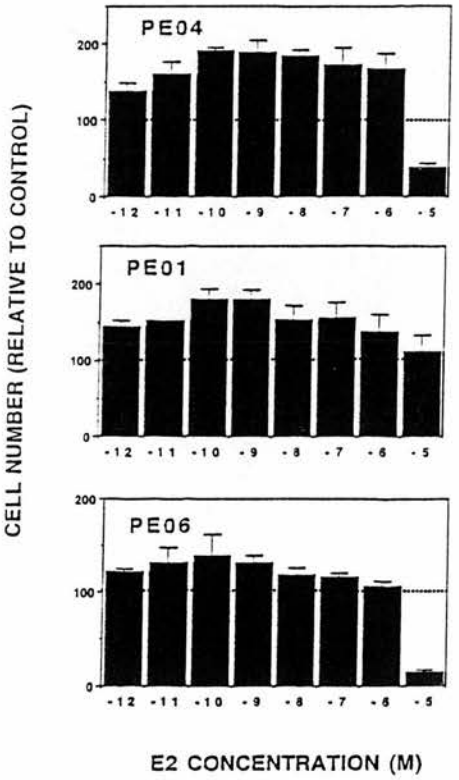

Fig. 1. Effect of 17 β-estradiol (E_2) on the growth of ER-positive ovarian carcinoma cell lines. Cells were treated for 6 days with E_2 (as described in Experimental) at the concentrations shown. Each value is the mean value of quadruplicate observations. Error bars represent standard error. The experiment shown is representative of at least 3 identical experiments.

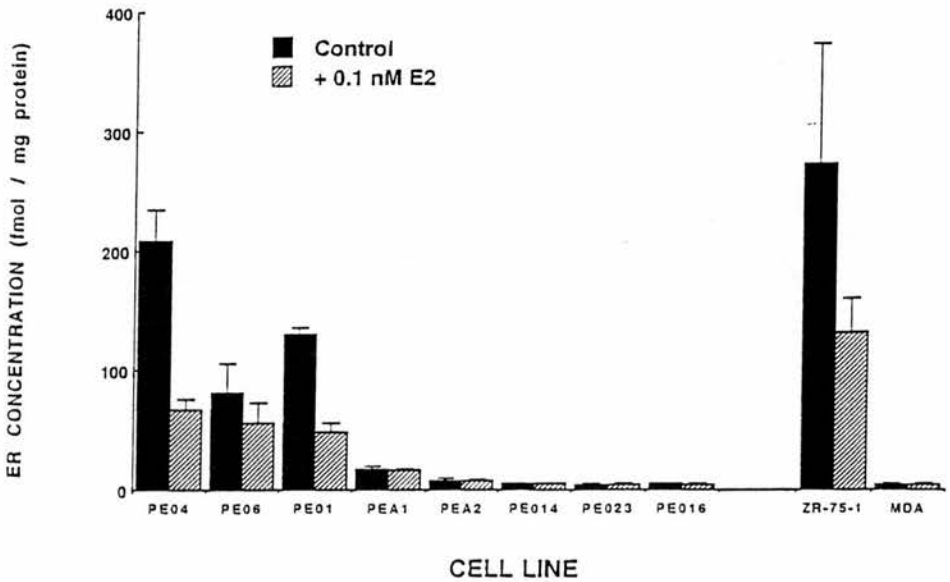

Fig. 2. Estrogen receptor content of ovarian and breast cancer cell lines in the absence and presence of 17 β-estradiol (E_2). Each point is the mean value of 3 independent measurements and error bars represent standard error. Solid bars represent values obtained on cells grown in the absence of E_2 and hatched bars are for cells grown in the presence of 10^{-10} M E_2 for 6 days (as described in Experimental).

not shown). Consistent with previous reports [22], growth of the ER-positive breast cancer ZR-75-1 cell line was stimulated by 10^{-10} M E_2 (120% increase in cell number relative to control) while growth of the ER-negative MDA-MB-231 line was unaffected (data not shown).

Effect of E_2 on ER concentrations

Two of the 3 ovarian carcinoma cell lines (PE04 and PE01) with high concentrations of ER demonstrated a reduction (approx. 50%) in the level of ER after exposure to 10^{-10} M E_2 compared to untreated cells (Fig. 2). This response was similar to that seen in the ZR-75-1 cell line (Fig. 2). The PE06 (81 fmol ER/mg protein) and the PEA1 (18 fmol ER/mg protein) lines did not show a significant reduction in ER content after exposure to E_2 compared to untreated cells. The other ovarian carcinoma cell lines examined possessed levels around the limit of detection (< 10 fmol/mg protein) in the absence or presence of 10^{-10} M E_2.

Effect of E_2 on PR concentrations

Two of the 3 ovarian carcinoma cell lines with high levels of ER (PE04 and PE06) showed an increase in the level of PR after exposure to 10^{-10} M E_2 compared to untreated cells (Fig. 3). The other ovarian lines demonstrated levels at the limit of detection of the assay. The ZR-75-1 line contained a much higher concentration of PR in the absence of E_2 (212 fmol/mg protein), than the ovarian carcinoma cell lines; PR was increased 5-fold after exposure to 10^{-10} M E_2.

Effect of E_2 on pS2 concentrations

The expression of pS2 in five of the ovarian carcinoma cell lines (PE04, PE01, PE06, PEA1 and PE014) was investigated in the presence or absence of 10^{-10} M E_2. No expression was found in these ovarian lines after either 3 or 6 days exposure to E_2. In contrast, the protein could be detected in the ZR-75-1 line and was increased by 260% in the presence of E_2 (data not shown). The ER-negative MDA-MB-231 breast cancer line did not express this protein.

DISCUSSION

This study indicates that E_2 can stimulate the growth of certain human ovarian carcinoma cell lines and this stimulation is associated with the presence of moderate–high levels of ER. These findings are similar to those found for breast cancer cell lines [23] and, as for breast cancer, suggest that the minimum requirement for ovarian tumour cells to be sensitive to estrogen is the presence of moderate numbers of ER. The ER value widely used to indicate likely sensitivity of breast cancer cells to anti-estrogen therapy (thus implying estrogen sensitivity) is 20–30 fmol/mg protein. To the best of our knowledge, no other ovarian cancer cell lines than those described in this report have been reported to have moderate–high levels of ER; therefore

these are the first systems which could act as a model for primary ovarian tumours with significant ER levels. Previous studies investigating the hormonal sensitivity of ovarian cancer have demonstrated that the BG-1 line ovarian carcinoma line which possesses an ER value of 23 fmol/mg protein is growth stimulated by E_2 [5] while the NIH:OVCAR-3 ovarian carcinoma line with an ER content of 28 fmol/mg protein is not [4] indicating that variable effects on growth are obtained around this cut-off point. A report describing clonogenic assays of human ovarian carcinoma cells obtained directly from clinical specimens indicated that 2 of 4 samples with an ER content greater than 30 fmol/mg protein responded to the anti-estrogen tamoxifen, while 0 of 14 samples with an ER value less than 30 fmol/mg were unresponsive, suggesting again that this value is a reasonable crude index of estrogen sensitivity [24]. Further experiments with clinical specimens of ovarian carcinoma are required to define a more accurate cut-off value.

Consistent with the view that E_2 is operating via the ER, levels of ER decreased in those ovarian carcinoma lines with the highest levels of ER after exposure to E_2 in a manner similar to that found for ER-positive breast cancer lines [10, 11].

For breast cancer, additional markers of estrogen response have been sought to help delineate more precisely that group of ER-positive tumors that are most likely to respond to anti-estrogen therapy. Among these are the PR and pS2 proteins [10–13]. In two of the three ER-positive ovarian carcinoma lines which were stimulated by E_2, PR was induced to a concentration of 20–30 fmol/mg protein. In the ZR-75-1 breast carcinoma line, PR levels in the absence of E_2 were 212 fmol/mg protein and this was increased to 920 fmol/mg protein after exposure to 10^{-10} M E_2. While the magnitude of the PR contents are markedly different between the ER-positive breast and ovarian carcinoma lines, the factor by which PR increases in the presence of E_2 is comparable in breast and ovarian cells. We have previously investigated the effects of exogenous E_2 on PE04 xenografts growing in nude mice and, in that system, the PR content increases from approx. 100 fmol to 800 fmol/mg protein after exposure to E_2 [3]. This increase, however, is associated with growth inhibition. The NIH:OVCAR-3 line also demonstrates an induction of PR after exposure to E_2; however, in this line there is no effect on growth [4]. Induction, therefore, of the PR in ER-positive ovarian carcinoma cell lines may indicate that protein expression is being modulated by estrogen, but does not imply that growth stimulation is also occurring. High levels of PR, therefore, in primary ER-positive ovarian tumours, may be indicative of a tumor being exposed to estrogen rather than a marker of estrogen sensitivity.

The expression of pS2 was investigated in several ovarian cancer cell lines. While expression of pS2 was detected in the ZR-75-1 breast line as previously reported, and was increased after exposure to E_2, no expression of this protein was found in the ovarian lines. Previous studies of primary ovarian tumours have identified pS2 in a subset of tumours [25–27]. Wysocki *et al.*[25] detected pS2 mRNA in 6 of 29 cases, 4 of

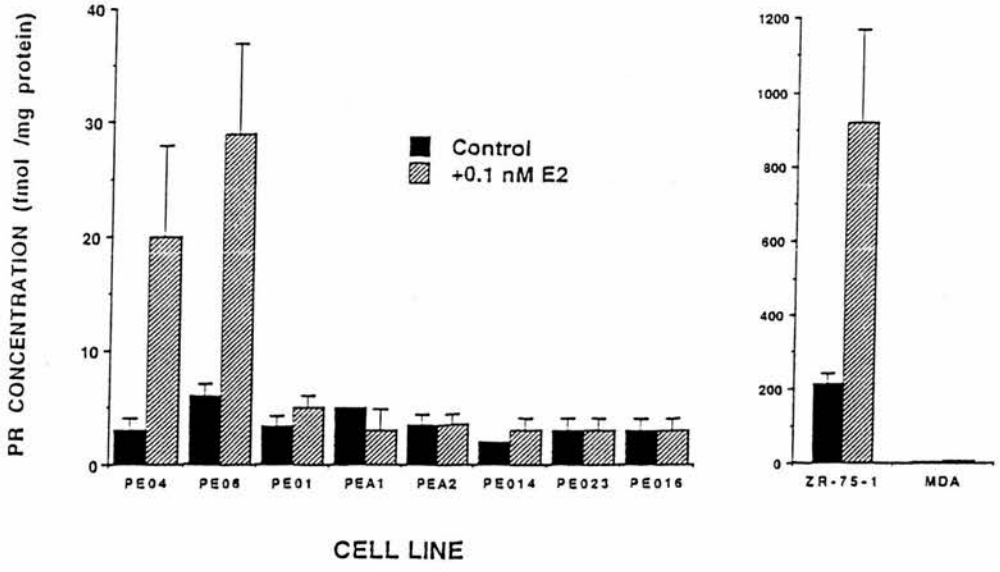

Fig. 3. Progesterone receptor content of ovarian and breast cancer cell lines in the absence and presence of 17 β-estradiol (E_2). Each point is the mean value of 3 independent measurements and error bars represent standard error. Solid bars represent values obtained on cells grown in the absence of E_2 and hatched bars are for cells grown in the presence of 10^{-10} M E_2 for 6 days (as described in Experimental).

which were of the mucinous subtype while Henry *et al.*[26] detected the protein (by immunohistochemistry) in 9 of 25 cases, of which 6 were also of the mucinous subtype, suggesting a possible association with this subtype. In contrast, Foekens *et al.*[27] using a radiometric immunoassay, were unable to detect levels above 11 ng/mg protein (the level they defined as clinically relevant) in 26 ovarian cancers. None of our cell lines was derived from a mucinous ovarian carcinoma but are derived from the serous subtype and thus our findings may be more representative of the "normal spectrum" of tumours. However, it is of interest, that in the study of Wysocki *et al.*[25], although 59% of the 29 tumours were ER-positive, 5 of the 6 demonstrating expression of pS2 had an ER concentration less than 20 fmol/mg protein suggesting that, in contrast to data for breast cancer, these two parameters are not linked. Indeed, the mucinous subtype, in contrast to the serous and endometriod forms of ovarian cancer, is more frequently ER-negative [1], again suggesting a probable lack of association of ER with pS2 in this disease.

In conclusion, we have demonstrated that the growth of a proportion of ovarian carcinoma cell lines can be stimulated by estrogen in culture. This stimulation appears to be associated with the presence of moderate–high levels of ER and may also be associated with the induction of PR. We are contributing to use these models to study other molecular pathways under control by estrogen to help identify indicators of hormonal sensitivity in clinical tumors.

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Expression of the Heat Shock Protein HSP27 in Human Ovarian Cancer[1]

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ABSTRACT

The relationship of the heat shock protein HSP27 in ovarian cancer to several biological and clinical parameters was investigated in a series of primary tumors and cell lines. Analysis of 72 primary tumors (54 malignant, 5 borderline, and 13 benign neoplasms) indicated that malignant tumors expressed higher HSP27 concentrations than benign tumors (median values, 0.56 *versus* 0.25 ng/μg cytosolic protein; $P = 0.032$). Tumors from patients with advanced stage (stages II, III, or IV) disease contained significantly higher HSP27 concentrations than tumors from stage I patients ($P = 0.018$), and an HSP27 content >2.0 ng/μg cytosolic protein was associated with reduced survival ($P = 0.03$). Tumors that had demonstrated progressive growth after chemotherapy had a significantly higher HSP27 content than tumors that were static or responsive ($P = 0.022$). These data indicate that HSP27 is associated with more aggressive malignant ovarian disease and with inherent resistance to chemotherapy.

Concentrations of HSP27 were also correlated with indicators of estrogen sensitivity. Therefore, the HSP27 concentration correlated with the estrogen receptor (all tumors, $P = 0.0014$; malignant tumors only, $P = 0.047$) but not with the progesterone receptor concentration. Analysis of ovarian cancer cell lines *in vitro* and *in vivo* indicated that the HSP27 content was higher in cell lines that were estrogen receptor rich and whose growth was modulated by estrogen as compared with those that were not. Additionally, two estrogen receptor-rich ovarian carcinoma lines demonstrated a small but significant decrease in HSP27 levels in response to 17β-estradiol in culture. These results suggest that HSP27 may help identify tumors responsive to estrogens.

INTRODUCTION

HSP27 is a M_r 27,000 member of the heat shock protein family which has previously been studied as p29 (1, 2), srp27 (3), and p24 (4, 5). In addition to its putative role in thermotolerance, it has been proposed to have a role in the development of drug resistance, to act as a molecular chaperone, and to be involved in signal transduction pathways (reviewed in Ref. 6). High concentrations of HSP27 have been associated with rapid proliferation in hormonally sensitive tissues, such as breast and endometrium, and relate to poor disease-free survival in breast cancer patients (7). Consistent with this view of HSP27 as a marker of proliferation, it is a negative prognostic factor in gastric cancer (8).

In breast and endometrial cancer, concentrations of HSP27 are qualitatively and quantitatively linked to the ER[3] (1, 2, 9). HSP27 was initially identified in the human breast tumor cell line MCF-7 as an estrogen-responsive protein (10), and subsequent studies in other ER-positive breast cancer cell lines have supported its potential as a biochemical marker of estrogen regulation (11, 12). Furthermore, the protein has been shown to be a predictor of clinical response to hormonal therapy in breast cancer (2, 13) and a marker of estrogenic response in normal and malignant endometrium (14–16). In cell line models of breast cancer, a good correlation has been demonstrated between the presence of ER and high amounts of HSP27 (9). The links between HSP27 and ER and between HSP27 and rapid proliferation in breast cancer are somewhat anomalous since hormonal sensitivity is generally associated with slow proliferation. However, in endometrial cancer, elevated levels of both ER and HSP27 are separately associated with good overall survival (17).

We are unaware of any previous reports of HSP27 in ovarian cancer, and in this article we describe the incidence of HSP27 in primary ovarian tumors and in ovarian cancer cells grown in culture or as xenografts. Furthermore, because evidence is accumulating to suggest that a proportion of ovarian adenocarcinomas are under endocrine control, we have investigated the relationship between HSP27, ERs, and estrogen-regulated growth in all of the aforementioned systems of ovarian cancer and have studied whether HSP27 is regulated by estrogen in ovarian cancer cell models *in vitro*.

MATERIALS AND METHODS

Cell Lines and Xenografts. The characterization of the ovarian cancer cell lines (PE04, PE06, PE01, PEA1, PEA2, PE016, PE014, and PE023) and xenografts (PE04-x and HOX 60) has been described previously (18–21). The PE01^{CDDP} line is a cisplatin-resistant subline derived from the PE01 line. Cell

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[3] The abbreviations used are: ER, estrogen receptor; PR, progesterone receptor; EIA, enzyme immunoassay; E_2, 17β-estradiol.

lines were routinely cultured at 37°C, 90% humidity, and 5% CO_2 in RPMI 1640 (GIBCO-BRL, Paisley, United Kingdom) containing 10% heat-inactivated FCS, streptomycin (100 μg/ml), and penicillin (100 IU/ml). The ZR-75–1 and MDA-MB-231 breast carcinoma cell lines were used as controls and were cultured in DMEM (GIBCO-BRL) supplemented with the above additives plus L-glutamine. The PE04-x and HOX 60 ovarian carcinoma and ZR-75–1 and T1068 breast carcinoma xenografts were maintained in the flanks of female nude (*nu/nu*) mice (obtained from OLAC, Oxford, United Kingdom) kept in negative pressure isolators (La Calhene, Cambridge, United Kingdom).

Primary Tumors. Tissue samples from 72 patients were collected at initial debulking surgery for suspected ovarian cancer. These were snap frozen in liquid nitrogen and stored at −180°C until use. Tumor histology was assessed on paraffin sections and classified according to WHO criteria (22). Tumors were classified as either malignant (54 tumors), borderline [low malignant potential (5 tumors)], or benign (13 tumors). Of the 54 malignant tumors, 25 were serous, 16 were endometrioid, 2 were mucinous, 5 were clear cell, 3 were mixed malignant mesodermal tumors, 1 was a steroid cell tumor, and 2 were mixed pathologies. The borderline group consisted of three mucinous and two serous subtypes. The benign group consisted of four mucinous adenomas, one serous adenoma, one Brenner tumor, two cystadenofibromas, one thecofibroma, three fibromas, and one tubo-ovarian cyst. Information on clinical stage was available for 52 of the 54 women with malignant tumors, and the group consisted of 13 stage I, 4 stage II, 33 stage III, and 2 stage IV tumors. Data on the histological grade were available for 51 of these tumors, and the group consisted of 2 well-differentiated, 16 moderately differentiated, and 33 poorly differentiated tumors. Survival data were available for 51 of 54 patients with malignant disease; postoperative chemotherapy was given to 37 of these 51 patients (5 received chlorambucil, 32 received cisplatin), while 14 patients with limited disease received no further treatment after surgery. A clinical evaluation of response to chemotherapy was available for 13 of the 37 treated patients. The criteria for response were as follows: complete response required the disappearance of all assessable disease on clinical and radiological examination and second-look laparotomy, clinical progression (or surgical progression at second-look laparotomy) constituted progressive disease, while static disease refers to no change at 6-month assessment.

Recombinant HSP27. Recombinant HSP27 was obtained from Sigma (Poole, United Kingdom). This was supplied lyophilized with phosphate buffer salts and sodium chloride, dissolved in distilled water, and then stored as aliquots at −80°C until used.

Preparation of Materials for ELISA. To determine the HSP27 concentrations in cell lines cultured *in vitro*, cell pellets were prepared as follows. Cells were grown in 10% FCS to late-log phase in 25-cm^2 flasks (Falcon, Cedex, France) and harvested by scraping, pelleted by centrifugation, and then stored at −80°C until use. In experiments where the effects of 17 β-estradiol were investigated, cells were grown to mid-log phase in 175-cm^2 flasks, washed with PBS, and media changed to phenol red-free media containing 5% double charcoal-stripped serum (23) plus penicillin, streptomycin, and glutamine. Twenty-four h later, cells were refed with the same media with or without 10^{-10} M 17 β-estradiol. After an additional 72 h, cells were collected by scraping and stored at −80°C. The cell pellets were resuspended in 0.5 ml Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, and 2 mM DTT; pH 7.4 at 4°C) and homogenized by hand. Samples were then spun at 200,000 × *g* for 1 h at 4°C, supernatants were collected for analysis, and pellets were discarded.

Human tumor xenografts, grown s.c. in the flanks of nude mice, were collected when mean diameters were 1 cm and stored in liquid nitrogen until use. Tumor material was homogenized in 5 volumes of Tris-EDTA buffer for 2 × 15 s in a Silverson homogenizer, centrifuged at 200,000 × *g* for 60 min, and stored at −80°C. Protein levels in the cytosol fractions were determined using a colorimetric assay adapted from Bradford (24).

ELISA Method for Measurement of HSP27. HSP27 expression was detected in sample cytosols by ELISA as described previously (13). Cytosols were diluted in PBS, plated in Immulon 4 flat-bottomed 96-well plates (Dynatech) at 200 μl/well, and incubated at 37°C for 90 min. The following protein concentrations were used: primary tumors, 2–10 μg/well; ovarian cell lines, 10–40 μg/well; and recombinant HSP27, 2–10 ng/well. Each concentration was examined in duplicate, and final values obtained were the mean values for three assays conducted on different occasions. Plates were washed three times with PBS-0.05% Tween 20 and then treated with or without the anti-HSP27 antibody D5 (0.15 μg in 200 μl PBS-Tween 20/well; Ref. 25) for 2 h at 37°C. After washing three times, wells were incubated with 200 μl/well peroxidase-conjugated antimouse immunoglobulin (DAKO, High Wycombe, United Kingdom) at 1:1000 in PBS-Tween 20 at 37°C for 1 h. Following additional washing, 200 μl of one of two peroxidase substrates were added for 4 to 10 min in the dark at 37°C. Primary tumor samples were treated with 0.5 mg *o*-phenylene diamine hydrochloride/ml substrate buffer (0.05 M sodium phosphate-0.02 M citric acid, pH 5.0) plus 8 μl 30% hydrogen peroxide. For the cell lines and xenografts with lower HSP27 levels, a more sensitive substrate was used: 100 μl tetramethyl benzidine (20 mg/ml DMSO)/20 ml substrate buffer plus 14 μl 30% hydrogen peroxide. The reactions were terminated with 50 μl/well 0.5 M sulfuric acid, and plates were read at either 492 nm (*o*-phenylene diamine hydrochloride substrate) or 450 nm (tetramethyl benzidine substrate) in a Bio-Rad plate reader. Background values (in the absence of D5) were subtracted, and comparison to a standard curve generated for recombinant HSP27 in each set of reactions yielded final values.

Measurement of ER and PR. Tissue fragments (50–200 mg) were weighed and homogenized in buffer (10 mM Tris, 0.25 M sucrose, 1 mM EDTA, pH 8.0, at 22°C, plus 1% monothioglycerol, and 10% v/v glycerol). After centrifugation at 105,000 × *g*, the supernatant cytosol was assayed by EIA using ER-EIA or PR-EIA kits (Abbott Ltd., Basingstoke, United Kingdom) according to the manufacturer's instructions. The protein content of the cytosol was determined as described above, and receptor concentrations were expressed as fmol/mg protein.

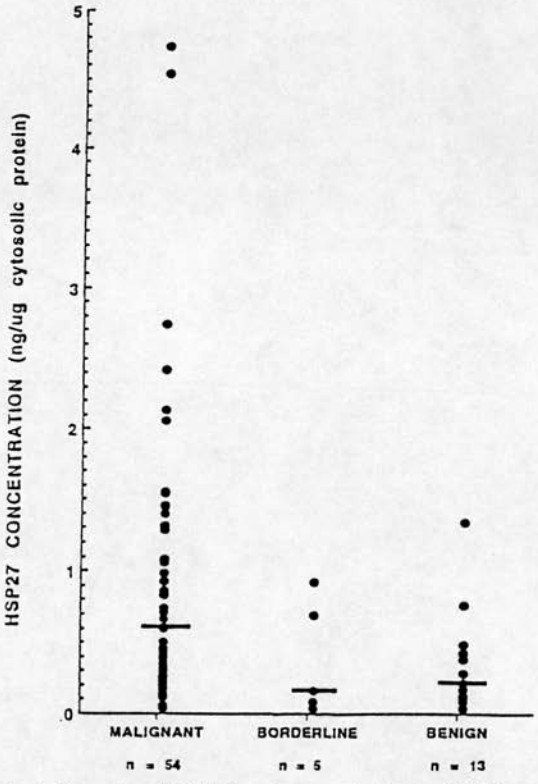

Fig. 1 Expression of HSP27 in primary ovarian tumors. HSP27 concentration was measured using an ELISA as described in "Materials and Methods." *Bars*, median values. Comparison of groups (Mann-Whitney *U* test): malignant *versus* benign, $P = 0.032$; malignant *versus* borderline; and borderline *versus* benign, nonsignificant.

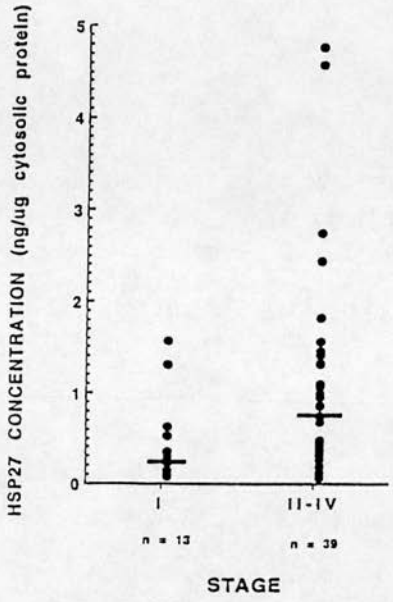

Fig. 2 Relationship betwen HSP27 expression and stage of ovarian cancer. HSP27 concentration was measured using an ELISA as described in "Materials and Methods." *Bars*, median values. Comparison of groups (Mann-Whitney *U* test): stage I *versus* stages II, III, and IV, $P = 0.018$.

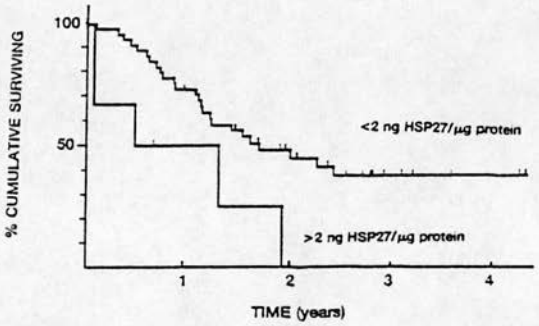

Fig. 3 Relationship between HSP27 expression and survival of patients with ovarian cancer. HSP27 concentration was measured using an ELISA as described in "Materials and Methods." Kaplan-Meier curves were compared using the log rank test, $P = 0.03$. *Ticks*, censored values.

RESULTS

HSP27 Expression in Primary Ovarian Tumors. The concentrations of HSP27 were measured using an ELISA in 72 primary ovarian tumors consisting of 54 malignant, 5 borderline, and 13 benign neoplasms. The measured values ranged from 0 to 4.7 ng HSP27/μg cytosolic protein (Fig. 1). Although there was overlap in the values between the malignant and other groups, the median value (0.56 ng/μg protein) for the malignant group was significantly higher than that of the benign group (0.25 ng/μg protein; $P = 0.032$, Mann-Whitney *U* test), whereas borderline tumors possessed a median value (0.13 ng/μg protein) comparable to that of the benign group.

HSP27 Expression and Clinicopathological Parameters in Malignant Tumors. Analysis of the malignant group by stage indicated that tumors of advanced stage (stages II-IV; *i.e.*, extending beyond the ovaries) possessed a higher median value (0.81 ng/μg protein) than stage I tumors (median, 0.22 ng/μg protein; $P = 0.018$, Mann-Whitney *U* test; Fig. 2). There were no significant associations between HSP27 content and either histological type or grade of differentiation (data not shown). Analysis of the survival of the group of patients with malignant tumors indicated that those patients ($n = 6$) with tumors containing an HSP27 concentration >2 ng/μg protein had a significantly poorer survival than the majority of patients ($n = 44$) who had a value <2 ng/μg protein ($P = 0.03$, log rank test; Fig. 3). Although this cutoff point was arbitrarily selected, it was noticeable that all patients with tumors above this value died before 2 years.

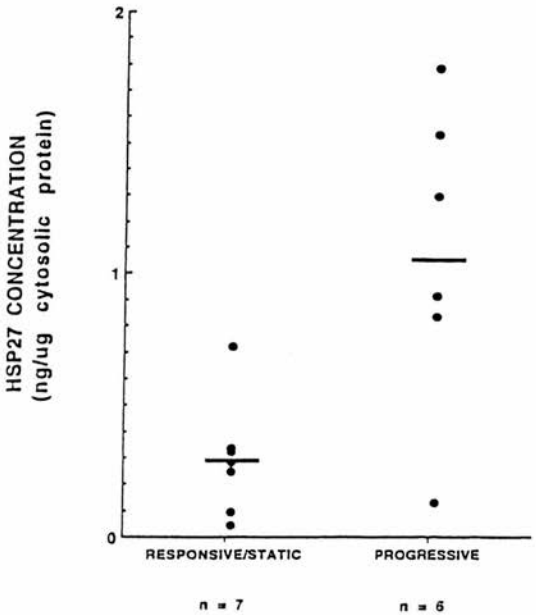

Fig. 4 Relationship between HSP27 and response to chemotherapy in ovarian cancer patients. HSP27 concentration was measured using an ELISA as described in "Materials and Methods." Criteria of response to chemotherapy are also described in "Materials and Methods." *Bars*, median values. Comparison of groups (Mann-Whitney *U* test): responsive or static tumors *versus* progressive tumors, $P = 0.022$.

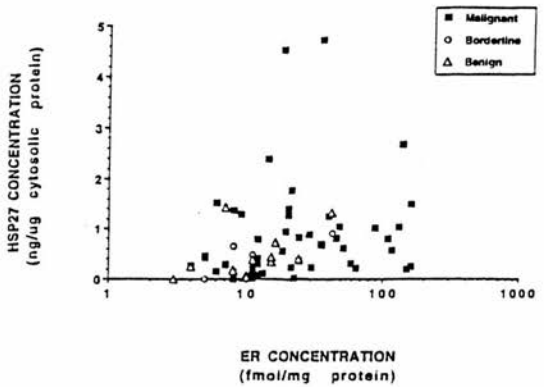

Fig. 5 Relationship between HSP27 and ER in primary ovarian tumors. HSP27 concentration was measured using an ELISA and ER using an EIA as described in "Materials and Methods." HSP27 and ER were correlated using the Spearman rank correlation: for all (*i.e.*, malignant, borderline, and benign) tumors, $P = 0.0014$; for malignant tumors only, $P = 0.047$.

HSP27 Expression and Response to Chemotherapy. The relationship between HSP27 content and response to chemotherapy was investigated in a subset of the 37 patients who had received treatment; evaluation of the response to chemotherapy was possible in only 13, of whom 7 tumors were "responsive" (2 complete responses and 5 static), while 6 progressed on chemotherapy. The HSP27 concentrations in these two groups of tumors are shown in Fig. 4, and the median value was significantly higher in the group of tumors that progressed compared to those that responded or remained static (1.09 *versus* 0.26 ng HSP27/μg protein; $P = 0.022$, Mann-Whitney *U* test).

HSP27 and ER and PR in Ovarian Tumors. The relationship between HSP27 and ER or PR was examined in a subset of 66 tumors in which data were available for ER and PR. A significant correlation was found between ER and HSP27 (all tumors, $P = 0.0014$; malignant tumors only, $P = 0.047$, Spearman's rank test; Fig. 5) ,but not between PR and HSP27 ($P = 0.97$; Spearman's rank test; data not shown).

HSP27 Expression in Ovarian Carcinoma Cell Lines *in Vitro* and *in Vivo*. The concentration of HSP27 was measured in nine ovarian carcinoma cell lines grown in 10% FCS-RPMI 1640 (Fig. 6). The values for these ovarian lines range from 0 to 3.8 ng HSP27/μg protein. The three ovarian lines (PE01, PE04, and PE06) with the highest levels (≥0.9 ng/μg protein) of HSP27 have previously been shown to be ER rich (>80 fmol/mg protein) and to be growth stimulated by E_2 (20). The cell lines PEA1, PEA2, PE016, PE014, and PE023 have lower levels (≤0.8 ng/μg protein) of HSP27; these lines have previously been shown to be ER poor (< 20 fmol/mg protein), and their growth is unaffected by the addition of E_2. The cell line $PE01^{CDDP}$ is a line derived from PE01, which has been made resistant to cisplatin. Although ER positive, it is growth inhibited by concentrations of E_2, which stimulate the growth of the other ER-positive cell lines, and in conditions of 10% FCS, it possesses a low HSP27 content.

The expression of HSP27 in two ovarian carcinoma xenografts was also investigated. The ER-positive PE04 xenograft, whose growth is modulated by E_2, possessed higher levels of HSP27 compared to the ER-negative HOX 60 xenograft, whose growth is unaffected by E_2 (Fig. 6). The HSP27 content of the PE04 xenograft was approximately 50% that of the cultured cell line (Fig. 6).

Regulation of HSP27 Expression by E_2. To assess whether HSP27 expression was under the regulation of estrogen in ovarian cancer cells, a range of cell lines were exposed to 10^{-10} M E_2 for 72 h. The ZR-75-1 breast cancer cell line was used as a positive control, in which exposure to E_2 increased expression of HSP27 (Fig. 7). In contrast, among the ovarian cell lines, only the PE01 line showed an increase (which was small and nonsignificant) after exposure to E_2, while the ER-positive PE04 and $PE01^{CDDP}$ lines demonstrated a significant reduction in their HSP27 content. HSP27 expression in the ER-negative PE014 ovarian and MDA-MB-231 breast lines was unaffected by exposure to E_2.

DISCUSSION

This is the first report on the expression of HSP27 in ovarian cancer, and the results show that ovarian tumors possess a range of values varying between 0 and 5 ng/μg cytosolic protein. In support of this protein having a role in the biology of

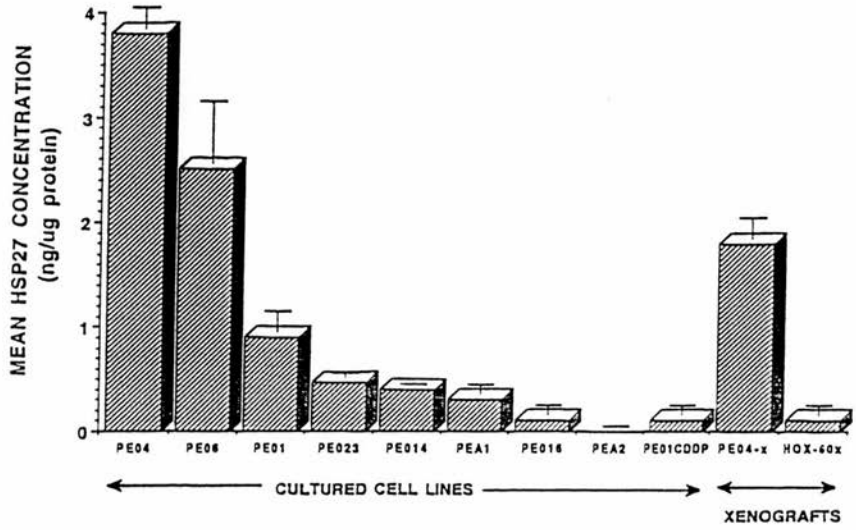

Fig. 6 HSP27 expression in ovarian cancer cell lines. HSP27 concentration was measured using an ELISA as described in "Materials and Methods." Values shown for the cultured cell lines are for cells grown in 10% FCS-90% RPMI 1640. Standard deviation shown.

this disease, a number of positive associations were observed between HSP27 concentration and pathological and clinical features in a series of primary ovarian tumors. First, malignant tumors as a group express significantly greater levels of this protein than benign tumors while borderline tumors have a level comparable to that of the benign group. This increase in concentration with malignancy is also consistent with the observation that HSP27 is absent in the normal ovary (4). It is possible that the increase in malignant tissues may be partially explained by increased cellularity, and to assess this and to also define which cell types are positive for HSP27, immunohistochemical studies will be needed.

Second, advanced stage tumors (stages II-IV) have a greater content of HSP27 than tumors limited to the ovary (*i.e.*, stage I tumors). Third, patients with tumors expressing higher levels of HSP27 (>2 ng/μg protein) had a significantly poorer prognosis than those patients with low tumor content of HSP27. This cutoff point was selected retrospectively and requires confirmation in a prospective cohort of patients. However, together these data indicate that increased expression of HSP27 is associated with aggressive disease. HSP27 has previously been shown to be associated with short disease-free survival in breast cancer (3, 7) and with poor survival in gastric cancer (8). Although HSP27 has not been previously investigated in ovarian cancer, the related protein HSP60 has been and also has been shown to be associated with a poor prognosis (26).

HSP27 has also been suggested to have a role in the drug resistance of tumor cells. For example, transfection of Chinese hamster ovary cells with HSP27 cDNA resulted in the development of resistance to a variety of antitumor agents (27). In the present study, HSP27 concentrations were significantly higher in tumors that progressed on therapy, as compared to those that were either static or clearly responsive. This is therefore compatible with the suggestion that high levels of HSP27 are associated with drug resistance.

In breast and endometrial cancer, HSP27 is also a predictor of endocrine sensitivity (2, 7, 9, 14, 16). In these tumor types, HSP27 correlates with ER but only weakly or not at all with PR (13, 14). The link between ER and HSP27 is not universal, and in, for example, squamous carcinomas of the uterine cervix or endometrial adenocarcinomas with squamous carcinoma cells, there is no association between HSP27 and ER (14). However, in the present study, HSP27 correlated with ER but not PR. To elucidate further whether there was a connection between HSP27 and estrogen sensitivity in ovarian cancer cells, the levels of HSP27 were measured in a range of cell line models whose sensitivity to estrogen had been defined previously (18–20). HSP27 concentrations were higher in the three lines that contained the highest levels of ER and whose growth was stimulated by E_2. It should be noted, however, that all three lines were derived from the same patient at different stages of clinical treatment and are therefore not truly independent (18). The five lines with low or negligible levels of ER and whose growth was unaffected by E_2 possessed lower levels of HSP27. The cell line $PE01^{CDDP}$ possesses a level of ER comparable to its parent cell line PE01. In contrast, however, its growth is inhibited by the addition of E_2.[4] This cell line contained a low concentration of HSP27 when grown in 10% unstripped serum conditions but demonstrated a value comparable to the other ER-positive cell lines when grown in 5% stripped serum conditions. In xenograft models of ovarian cancer, HSP27 content was higher in an ER-positive xenograft whose growth was modulated *in vivo* by

[4] Unpublished data.

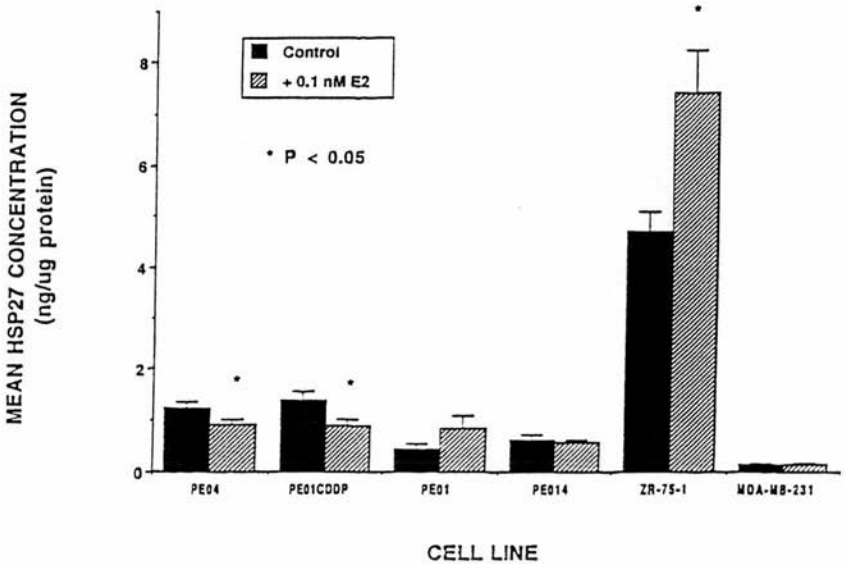

Fig. 7 Modulation of HSP27 by E_2 in ovarian cancer cell lines. HSP27 concentration was measured using an ELISA as described in "Materials and Methods." The ER-positive ZR-75-1 and ER-negative MDA-MB-231 breast carcinoma cell lines were included as controls. Untreated (control) cells were grown in 5% double charcoal-stripped serum in phenol red-free media (RPMI 1640 for the ovarian cancer lines and DMEM for the breast cancer lines). Estrogen-treated cells were exposed to E_2 (0.1 nM) for 72 h as described in "Materials and Methods. *, comparison of E_2-treated and untreated groups (*t* test), $P > 0.05$. Standard deviation shown.

E_2 compared to an ER-negative, unresponsive xenograft. These data with cell lines, *in vitro* and *in vivo*, are similar to a previous report for breast cancer cell lines in which the six ER-positive breast cancer cell lines studied contained a higher HSP27 content than two ER-negative breast lines and nine ER-negative nonbreast cancer cell lines (9). Additional evidence of a connection between ER and HSP27 was obtained by comparing the HSP27 concentrations in cells treated with or without E_2. Only cell lines with moderate to high concentrations of ER demonstrated a response, although, interestingly, the HSP27 concentration increased in the ER-positive ZR-75–1 breast line but decreased in two of the ER-positive ovarian cell lines. Previous studies of estrogen-sensitive systems have demonstrated that E_2 increases HSP27 levels in the ER-positive MCF-7 and ZR-75–1 cell lines (4, 11, 12) but not in the T47D line (9).

This is the first report of the expression of HSP27 in ovarian cancer, and these data indicate that HSP27 has both biological and clinical significance in this disease. In addition to being associated with poor prognosis and drug resistance, HSP27 is linked to the ER status and may help define endocrine-sensitive tumors.

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Growth control of human ovarian carcinoma cells by insulin-like growth factors

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Abstract. The role of the insulin-like growth factors (IGFs) in 3 cultured human ovarian cancer cell lines (PEO1, PEO4, PEO14) was investigated. All three cell lines express mRNA for IGF-I and the PEO14 cell line expresses mRNA for IGF-II. Protein expression of IGF-II was demonstrated in the PEO14 and PEO4 cell lines. All 3 cell lines expressed mRNA for the IGF type I, IGF type II and insulin receptors; the presence of type I IGF receptors was confirmed by immuno-cytochemistry. IGF-I and insulin markedly stimulated the proliferation of PEO1 and PEO4 but not PEO14 cells while all 3 lines were insensitive to the addition of exogenous IGF-II.

Introduction

There is an increasing awareness of the importance of polypeptide growth factors in the control of proliferation in cancers. In this respect, both the TGF-α/EGF and TGF-ß growth factor families have been shown to influence the growth of ovarian cancer cells in culture (1-5). To date, limited information is available on the role of the insulin-like growth factors (IGFs) in this important gynecological cancer (6-9). Nevertheless, the IGFs have been widely implicated in the paracrine control of normal ovarian function, acting both as local growth regulators and modulating the actions of pituitary gonadotrophins on the various cell types found in the normal ovary (10-12). IGFs have also been suggested to have a role in the growth control of breast cancer (13-15). The distribution of IGFs in breast tumor tissues implies that their primary role in this tumor may be paracrine (16,17), but reports that breast cancer cell lines express IGFs and their receptors *de novo* suggests that autocrine regulation cannot be wholly ruled out (16).

These observations, plus recent reports that indicate that IGF-I and its receptor are widely expressed in ovarian cancer models (6) and that type I IGF receptors may be present in ovarian tumors (18-22) have provided the impetus for the current study. The aim was to establish whether members of the IGF family play a role in regulating proliferation of ovarian carcinoma cell lines *in vitro*.

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Key words: ovarian cancer, insulin-like growth factors

Materials and methods

Cell lines. The human ovarian carcinoma cell lines PEO1, PEO4 and PEO14 were established and characterised as described previously (23). They were maintained routinely at 37°C in a humidified atmosphere of 5% CO_2 in air in RPMI 1640 (Gibco) containing 10% heat-inactivated fetal calf serum (FCS) supplemented with streptomycin (100 µg/ml), penicillin (100 IU/ml) and glutamine (2 mM).

Growth experiments. Exponentially growing cells were harvested by trypsinisation and plated in 24-well plates (Corning) at densities of 2.5×10^4 cells /well (for PEO1 and PEO4) or 5×10^4 (for PEO14) (4 wells per experimental condition) in RPMI 1640 containing 10% heat inactivated fetal calf serum (FCS). After 24 h, medium was removed, cells were washed with phosphate buffered saline (pH 7.4; PBS) and medium replaced with RPMI 1640 (phenol red free) containing either 5% double charcoal stripped-FCS (DCS-FCS), 0.5% DCS-FCS, HTS (hydrocortisone; 10 nm, transferrin 10 µg/ml, sodium selenite; 30 nm) or HTS containing 5 µg/ml insulin (HITS); and incubated for a further 24 h. Medium was then removed and replaced with RPMI with the corresponding additives (as above) with or without human insulin, recombinant IGF-I or IGF-II (Boehringer) added at concentrations ranging from 10^{-7} to 10^{-10} M. This time point was designated day 0. Media was replenished on day 3. On days 0, 3, and 6, cells were harvested by trypsinisation and counted using a Coulter counter.

mRNA extraction. mRNA was extracted as previously described (2), using the lithium chloride/urea method.

Synthesis of riboprobes. Labelled RNA was prepared from linearised template DNA using a Gemini II system (Promega Ltd, Southampton, UK) (see ref. 2) in the presence of an RNAse inhibitor, cold ribonucleosides, dithiothreitol and ^{32}P-rCTP with the appropriate RNA polymerase. The DNA template was then removed using RQ1 DNase. Labelled RNA was precipitated in the presence of added tRNA and full

length transcripts were isolated by polyacrylamide gel electrophoresis and labelled RNA eluted from the gel, precipitated under ethanol and resuspended in hybridisation buffer prior to use in RNAse protection assays. The IGF-I and IGF-II riboprobes were kindly supplied by Dr Jim Scott (CRC, Middlesex, UK).

RNAse protection assay. Test RNA (20 μg) was precipitated under ethanol, dried and resuspended in 30 μl hybridisation buffer (80% formamide, 40 mM Pipes (pH 6.7), 400 mM NaCl, 1 mM EDTA); tRNA was used as a negative control. Test probes plus actin probe were added to each sample. Samples were incubated at 85°C for 20 min and hybridized overnight at 51°C. After hybridization, single stranded RNA was removed using an RNAse A and T1 mix, followed by incubation with proteinase K in SDS. Protein was extracted by using phenol-chloroform-isoamyl alcohol. Protected RNA was precipitated with carrier tRNA (5 μg) and separated by gel electrophoresis. Full length transcripts for test probes were scored as positive, whilst transcripts for actin were used as an internal control.

Reverse transcriptase-polymerase chain reaction. Reverse-transcriptase polymerase chain reactions for type I and type II IGF receptors were carried out using a Techne PHC-3 thermal cycling block. Essentially, 20 μg aliquots of total cellular RNA were reverse transcribed by incubation with Superscript (Life Technologies, Paisley, UK) reverse transcriptase (200 units) for 1 h at 42°C. Subsequently 0.2-1 μl of this reaction product was utilized for PCR reactions. PCR reactions were performed in a volume of 100 μl, 0.5 units of TAQ polymerase (Promega, Southampton, UK) were combined with 1.25 μM dATP, dTTP, dCTP and dGTP (Pharmacia, UK), 100 ng of each primer in buffer containing KCl 50 mM, Tris-HCl 10 mM, Triton X-100 0.1% with added $MgCl_2$ 2.5 mM. The amplification reaction was carried out over 40 cycles with the following parameters: step 1: 94°C for 38 seconds, step 2: 50°C for 53 seconds, step 3: 72°C for 68 seconds. For the final cycle the 72°C step was extended to 7 min to ensure all transcripts were full length. The following primers were used in these reactions: Insulin receptor: 407 bp fragment spanning bases 515-922 of the insulin receptor mRNA (24); type I IGF receptor: 549 bp fragment spanning bases 1856 to 2405 of the type I IGF receptor mRNA (25) type II IGF-receptor: 394 bp fragment spanning bases 787 to 1181 of the type II IGF receptor mRNA (26).

Immunocytochemical detection of type I IGF receptor. The presence of type I IGF receptors was detected using an immunoperoxidase method employing avidin-biotinylated horseradish peroxidase complex (27); cells were trypsinised, washed with serum-free RPMI 1640, and placed onto multispot slides (Hendley Ltd, Essex, UK) at approximately $2x10^4$ cells/spot. These were then fixed in acetone:methanol (1:1) for 5 minutes and stored at -20°C until use. Slides were thawed and incubated for 10 minutes at room temperature with 3% hydrogen peroxide in methanol. The slides were then washed in 0.05 M Tris Buffer (TB), pH 7.6 and incubated with rabbit serum (Dako) in TB (1:5) for 20 minutes followed by incubation with the mouse monoclonal antibody (α-IR-3, 100 μg IgG/ml diluted 1:5 with TB; Oncogene Science) for 30 minutes. A further wash in TB was followed by incubation for 30 minutes with biotinylated rabbit anti-mouse immunoglobulin (Dako) diluted in TB (1:200). After washing, AB complex (Dako) was applied to cells and left for 30 min. A final TB wash was given before peroxidase was localised using a fresh 1 mg/ml mixture of 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.01% hydrogen peroxide in Tris imidazole buffer, pH 7.6 for 10 minutes. After washing with water, cells were counterstained with hematoxylin and scored for the presence and intensity of positive staining. TB was included in each staining run as a negative control and the monoclonal antibody CAM 5.2 which reacts against cytokeratin as a positive control.

Statistics. Cell growth and cell cycle responses *in vitro* were analyzed using the Wilcoxon Rank test and significant differences at the $p<0.05$ level defined.

IGF-II protein expression. Cells were grown to late-log phase in 175 cm^2 flasks. Media (50 ml: either 5% DCS-FCS in RPMI 1640 or RPMI 1640 alone) conditioned by 48 h exposure to PEO4 or PEO14 cells was collected and concentrated 25-fold. 10 μg of each sample was separated by electrophoresis on a 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis gel. Proteins were transferred to nitrocellulose overnight. These filters were then immunoblotted using a monoclonal antibody to IGF-II (Sera-Lab, Sussex, England). Detection was by enhanced chemoluminescence with exposure of the film for 15 seconds.

Results

mRNA expression for IGFs and IGF-receptors in ovarian carcinoma cell lines. All three cell lines tested (PEO1, PEO4 and PEO14) expressed high levels of mRNA for IGF-I (Fig. 1a). Expression of mRNA for IGF-II could be detected in PEO14 but not in either PEO1 or PEO4 cells (Fig. 1b). All three cell lines expressed mRNA for the insulin receptor and type I and II IGF receptors as determined by RT-PCR (Fig. 2).

Protein expression of IGF-II. The presence of IGF-II was investigated in conditioned media obtained from PEO4 and PEO14 cells. In the presence of 5% DCS-FCS, both cell lines express IGF-II, though PEO14 cells express much greater quantities (Fig. 3). PEO4 cells express primarily the mature 7 kDa form of the peptide with small amounts of a 15 kDa pro IGF-II form. PEO14 cells express greater quantities of both these forms of IGF-II than PEO4, together with an additional band at 24 kDa probably representing prepro IGF-II. Small amounts of the 7 kDa form were found in the serum control but the levels detected in the medium from PEO4 cells were greater. In conditioned media, collected in the absence of serum, a much smaller quantity of IGFII (than found in serum-containing conditions) could be detected in the PEO14 samples but not in the PEO4 samples.

Immunocytochemistry of type I IGF receptor. Over 90% of cells from all three cell lines stained positively for the type I IGF receptor as determined using the α-IR-3 monoclonal antibody (data not shown).

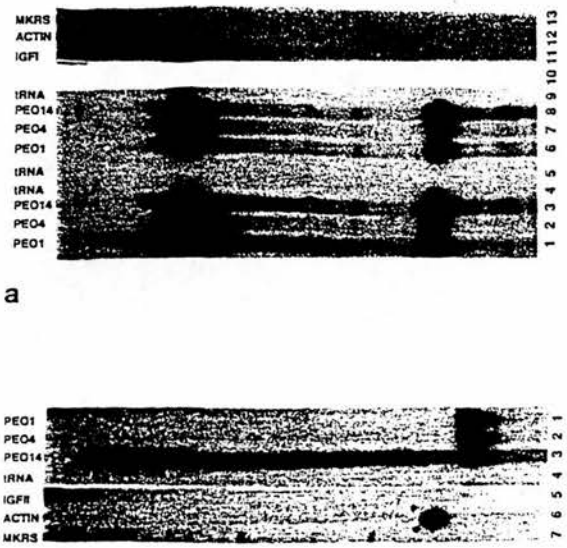

Figure 1. IGF-I and IGF-II mRNA expression detected by RNAse protection assay. 6% polyacrylamide gels, showing bands representing mRNA from IGF-I, IGF-II and human-c-actin. Panel A, IGF-I expression. Lane 13 contains ^{35}S labelled molecular weight markers; Lanes 11 and 12 contain untreated riboprobes for IGF-I and actin respectively; Lane 4-5 and 9 contain tRNA as a negative control; Lanes 1-3 and 6-8 contain test samples. mRNA from all three cell lines showed positive hybridization with IGF-I riboprobe. Panel B, IGF-II expression. Lane 7 contains ^{35}S labelled molecular weight markers; Lanes 6 and 5 contain untreated riboprobes for actin and IGF-II respectively; Lane 4 contains tRNA as a negative control; Lanes 1-3 contain test samples. Only mRNA from the PEO14 cell line showed positive hybridization with IGF-II riboprobe.

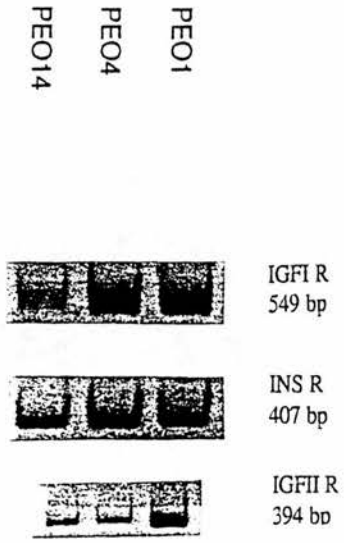

Figure 2. Insulin-, type I- and type II IGF receptor mRNA expression detected by RT-PCR. 6% polyacrylamide gels, showing bands representing mRNA from type I IGF receptor, type II IGF receptor and insulin receptor. Top row, mRNA from PEO14 (lane 1), PEO4 (lane 2) and PEO1 (lane 3). All cell lines show a band at approx 549 base pairs equivalent to the type I IGF receptor. Middle row, mRNA from PEO14 (lane 1), PEO4 (lane 2) and PEO1 (lane 3). All cell lines show a band at approx 407 base pairs equivalent to the insulin receptor. Bottom row, mRNA from PEO14 (lane 1), PEO4 (lane 2) and PEO1 (lane 3). All cell lines show a band at approx 394 base pairs equivalent to the type II IGF receptor. The primers used to detect these receptors are described in Materials and methods.

Growth responsiveness of PEO1 ovarian carcinoma cell line. The addition of IGF-I to cultures of PEO1 cells growing in serum-free conditions produced significant, dose related (range 10^{-7}-10^{-10} M) stimulation of growth after both 3 (data not shown) and 6 days in culture (Fig. 4a). Addition of insulin at the concentration widely used in serum-free media, $7x10^{-7}$ M (5 µg/ml), did not alter the effects produced by IGF-I but growth stimulation was less marked in the presence of 0.5% DCS-FCS and absent in the presence of 5% DCS-FCS (data not shown). Addition of exogenous IGF-II over the dose range 10^{-7}-10^{-10} M to the PEO1 ovarian cell line produced no detectable effect on cell growth after 6 days culture in serum free medium (Fig. 4b); again addition of insulin or DCS-FCS did not alter this response (data not shown). Insulin alone produced significant, dose related (range 10^{-7}-10^{-10} M) stimulation of growth of PEO1 cells under serum-free conditions after both 3 (data not shown) and 6 days in culture (Fig. 4c). Growth stimulation was also seen when cells were cultured in the presence of 0.5 or 5% DCS-FCS (data not shown).

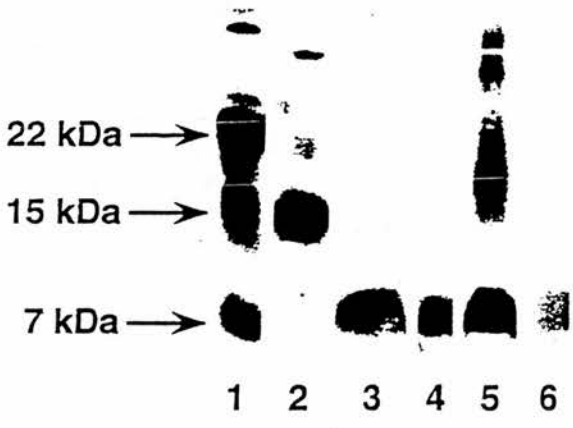

Figure 3. IGF-II peptide expression in conditioned media from the PEO4 and PEO14 cell lines. Immunoblot with monoclonal antibody to IGF-II of conditioned media (CM) followed by enhanced chemoluminescence for 15 seconds. Lane 1: CM from M17 cell line transfected with IGF-II cDNA contains 3 major forms of IGF-II at 22, 15 and 7 kDa; Lane 2: Molecular weight markers; Lane 3: 20 ng mature IGF-II; Lane 4: CM from PEO4 cells grown in serum; Lane 5: CM from PEO14 cells grown in serum; Lane 6: Control medium containing serum in the absence of cells.

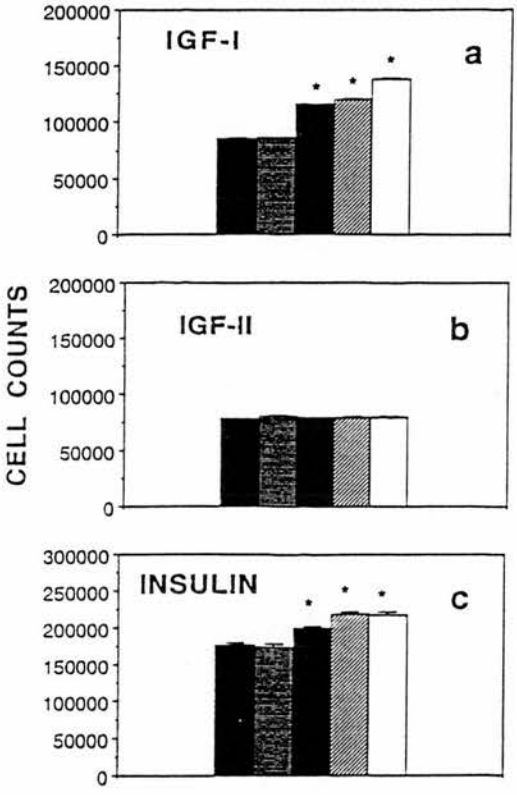

Figure 4. Growth responses of PEO1 ovarian carcinoma cells. Cell counts per well of PEO1 ovarian carcinoma cells in serum-free and insulin-free RPMI containing HTS. Solid bars represent untreated cells. Each point represents mean ± S.E. of quadruplicate points. Solid = untreated cells; horizontal = 10^{-10} M, hatched = 10^{-9}, stripes = 10^{-8} M, open = 10^{-7} M; cells exposed to 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} M growth factor respectively over 6 days. *Statistically significant difference with respect to time matched control ($p<0.05$). Panel a: Cells exposed to exogenous IGF-I. Panel b: Cells exposed to exogenous IGF-II. Panel c: Cells exposed to exogenous insulin (see text for details).

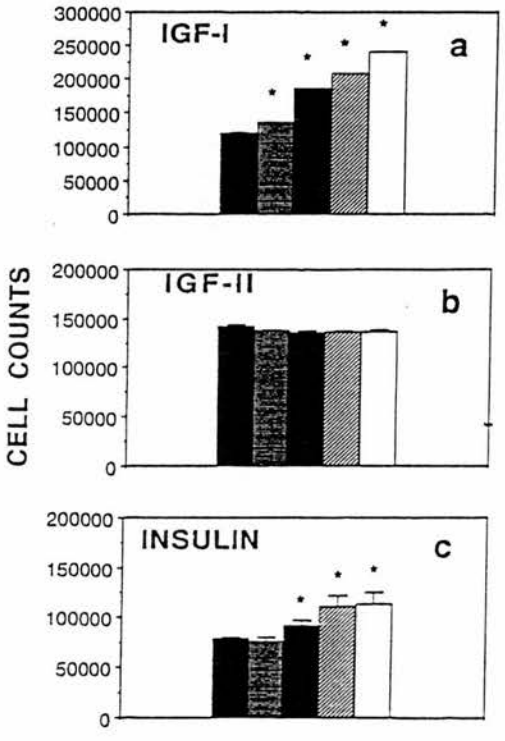

Figure 5. Growth response of PEO4 ovarian carcinoma cells: Cell counts per well of PEO4 ovarian carcinoma cells in serum-free and insulin-free RPMI containing HTS. Solid bars represent untreated cells. Each point represents mean ± s.e. of quadruplicate points. Solid = untreated cells; horizontal = 10^{-10} M, hatched = 10^{-9} M, stripes = 10^{-8} M, open = 10^{-7} M; cells exposed to 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} M growth factor respectively over 6 days. *Statistically significant difference with respect to time matched control ($p<0.05$). Panel a: Cells exposed to exogenous IGF-I. Panel b: Cells exposed to exogenous IGF-II. Panel c: Cells exposed to exogenous insulin (see text for details).

Growth responsiveness of PEO4 ovarian carcinoma cell line. IGF-I produced a significant, dose related (range 10^{-7}-10^{-10} M) stimulation of growth of PEO4 cells under serum-free conditions after both 3 (data not shown) and 6 days in culture (Fig. 5a). Addition of insulin (7×10^{-7} M) to serum free-media did not alter the effects observed but growth stimulation was not observed in the presence of either 0.5 or 5% DCS-FCS (data not shown). Addition of exogenous IGF-II over the dose range 10^{-7}-10^{-10} M to the PEO4 ovarian cell line produced no detectable effect on cell growth after 6 days culture in serum-free medium (Fig. 5b); addition of insulin or DCS-FCS did not alter this response. Insulin produced significant, dose related (range 10^{-7}-10^{-10} M) stimulation of growth of PEO4 cells under serum-free conditions after both 3 (data not shown) and 6 days in culture (Fig. 5c). Growth stimulation was also seen when cells were cultured in the presence of 0.5 or 5% DCS-FCS (data not shown).

Growth responsiveness of PEO14 ovarian carcinoma cell line. No significant response was observed in PEO14 cells exposed to exogenous IGF-I, IGF-II or insulin in either the absence or presence of insulin (IGF-I and -II only), 0.5 or 5% DCS-FCS (data not shown).

Discussion

Using three established cell lines, the growth regulation of ovarian carcinoma cells by IGFs has been investigated. High levels of mRNA expression for IGF-I were found in all three cell lines, along with mRNA for the type I IGF receptor. Immunocytochemical analysis of these cell lines confirmed that the type I IGF receptor was expressed in all three lines. Following incubation with exogenously added IGF-I, significant increases in the rates of cell proliferation were noted in both the PEO1 and PEO4 cell lines, whilst proliferation in the PEO14 cell line was not markedly affected. Taken together, these data

suggest that IGF-I may act as a regulator of proliferation in some ovarian carcinoma cell lines. Similar growth response and receptor data were obtained for insulin.

Of the three cell lines tested, only the PEO14 cell line showed expression of mRNA for IGF-II using RNAse protection methodology. The production of this peptide by this cell line was confirmed by Western blotting which demonstrated the presence of at least 3 forms of the peptide consistent with prepro-, pro- and mature IGF-II. The presence of the larger forms may be indicative of saturation of processing. The increased production of this peptide in serum-containing compared to serum-free conditions suggests that serum factors are involved in IGF-II production/secretion; alternatively, binding proteins in serum might increase the half-life of the peptide or might sequester the peptide from the type I receptor, thus preventing internalisation. IGF-II peptide was also produced by PEO4 cells, although the mRNA was not detected.

This may be due to the long conditioning period used for collection of the peptide allowing sufficient quantities to accumulate for detection. This endogenous production of IGF-II by the cell lines may provide maximal stimulation within the cells and prevent a growth response to exogenous factor.

The variation in growth responsiveness between cell lines may reflect either changes in levels of endogenous production of IGFs or may be due to alterations in the post-receptor transduction of the IGF mitogenic signal. It is also possible that the production of IGF binding proteins may alter the response of cells to IGF (28-33) since at least five IGF binding proteins are produced by PEO4 cells (7).

The finding that the effect of IGF-I is progressively diminished by addition of serum may be explained if it is assumed that binding proteins present in serum are sequestering IGF and preventing its action upon its receptor; alternatively, IGF-I within serum may mask any growth effect. Studies in a lung cancer cell line suggest that the presence of IGF binding proteins of different types may affect the ability of the IGFs to bind to receptors and to induce proliferative responses; some binding proteins may preferentially block the action of IGF-II allowing normal responses to IGF-I to persist (Reeve *et al*, Proc AACR 33: (Abst) 71, 1992). Such observations might account for the differences in effects of IGF-I and IGF-II observed here.

These data support a role for the IGF family in the growth regulation of ovarian carcinoma and confirm and extend previous studies which have demonstrated that IGF-I and its receptor are not only present in ovarian cancer but are responsive to modulation by the growth factor and anti-sense oligodeoxynucleotide inhibition (6,8). As such these pathways provide possible targets for therapy. The type-I, type-II and insulin receptors all possess tyrosine kinase activity and a great deal of attention is currently focussing on the therapeutic potential of tyrosine kinase inhibitors as antitumor agents (34). Interruption of these pathways may prove a beneficial approach in this disease.

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